IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of: Hong Jin *et al.*

Application No.: 09/724,788

Filed: November 28, 2000

For: RECOMBINANT RSV EXPRESSION SYSTEMS AND VACCINES

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Group Art Unit: 1648

Examiner: Lucas, Zachariah

Attorney Docket No.: 7682-051-999

DECLARATION OF RICHARD R. SPAETE UNDER 37 C.F.R. § 1.132

I, Richard R. Spaete, of Emerald Hills, California, do declare and state that:

1. I am currently a Senior Director, Research, at MedImmune Vaccines Inc., Mountain View, CA, and on the Editorial Board of the Journal of Virology. I was awarded a Ph.D. degree in 1982 from the University of Chicago's Committee on Virology, Chicago, IL. Upon completion of my doctorate, I worked as a Postdoctoral Fellow in the laboratory of Dr. Niza Frenkel (Committee on Virology faculty member), at the University of Chicago, Chicago IL and then as a Postdoctoral Fellow in the Department of Microbiology at Stanford University, Palo Alto, CA, where I was the recipient of both a Dean's Fellowship award and a Leukemia Research Foundation Postdoctoral award. In 1986 I joined the Chiron Corporation as a Scientist, I was subsequently promoted to Principle Scientist in 1986 and to Senior Scientist in 1992. I joined Aviron, Burlingame, CA in 1992 as a Senior Scientist, was promoted to Director, and remained with the company as a Director and now as a Senior Director when it was acquired by MedImmune, Inc.

2. During my entire career I have been working in the field of virology with a specific focus on the biology of herpesviruses, including herpes simplex virus (HSV), human cytomegalovirus (hCMV), Epstein Barr virus; orthomyxoviruses, such as influenza virus; and paramyxoviruses including parainfluenza virus (PIV), respiratory syncytial virus (RSV), and more recently human metapneumovirus (hMPV). The scope of my work has encompassed the generation and production of viral amplicons, and the generation of recombinant viruses and vaccines. My work in the fields of HSV and hCMV virology are detailed in numerous patents and scientific publications which include, among others, U.S. Patents No. 5,474,914; 5,547,834; 5,922,857; 5,721,354; 5,767,250; and 5,834,307. I have also co-authored two textbook chapters on virological subjects. My contributions to the fields of HSV and CMV virology have been acknowledged by numerous grant awards and invitations to speak at scientific meetings. A copy of my Curriculum Vitae is included with this declaration as Exhibit A.

3. I have reviewed patent application no. 09/724,388 entitled "Recombinant RSV Expression Systems and Vaccines" (the "'388 application"). I have been asked to evaluate whether, in the mid- to late 1990s, a molecular virologist, following the teachings and guidance set out in the '388 application, would, using ordinary skill, be able to (a) engineer mutant infectious, replication competent viruses belonging to the paramyxovirus family, in particular, Respiratory Syncytial Virus ("RSV"); and (b) generate vaccine strains of these viruses.

BACKGROUND.

4. Before beginning my analysis of the '388 application, a little background on vaccine development for RSV disease will be beneficial. Human RSV is an agent of serious

respiratory tract disease. Infection by RSV is one of the largest contributors to mortality in the very young and the very old (reviewed in Thompson et al. 2003, JAMA 289:172). RSV infections occur seasonally; the virus typically circulates during the winter months. Infection causes symptoms such as fever, rhinorrhea, coughing, wheezing, bronchiolitis, and pneumonia. Despite the induction of a neutralizing immune response to natural infection with wild-type virus, seasonal re-infection is a hallmark of RSV disease – almost all children become infected by two years of age, and 50% become re-infected (Byrd & Prince, 1997, Clin Infect Dis. 25:1363-1368; "Byrd & Prince"). Since the immunity which results from natural infection with wild-type virus does not necessarily prevent re-infection, no vaccine could be expected to completely prevent infection or re-infection. Instead, the goal of an RSV vaccine is to induce an immune response that ameliorates the disease consequences associated with initial infection and re-infection, by reducing the viral titer in the infected subject.

5. In a clinical trial conducted in 1966-1967, a formalin-inactivated RSV vaccine was administered to children and infants. Unfortunately, infants that had been seronegative for RSV at the time of vaccination developed enhanced disease symptoms during subsequent natural re-infection. The rate of hospitalization was increased among these infants, and two died. This bad experience with the formalin-inactivated RSV trials hampered vaccine development for RSV disease (reviewed in Byrd & Prince).

6. By the mid-1980s, an important animal model -- called the cotton rat model -- was developed to study RSV disease and to test vaccine candidates for RSV disease potentiation (reviewed in Byrd & Prince). Cotton rats are semi-permissive for RSV infection, and can be used to assess levels of viral replication and disease. More

importantly, however, the cotton rats can develop vaccine-enhanced pulmonary disease that models the human disease. Indeed, the vaccine-enhanced disease observed with the formalin-inactivated vaccine human trial was reproduced in the cotton rat. As a result, the cotton rat model became the primary animal model for testing RSV vaccine safety.

7. In addition to the cotton rat, a number of animal models, including small animals (rodents) and non-human primates (African green monkeys, and chimpanzees), were developed for testing attenuation and activity of RSV vaccine candidates (reviewed in Byrd & Prince). Typically, these models were used to measure replication of the vaccine strain relative to wild-type virus (as an assessment of attenuation); as well as induction of an immune response, and reduction in viral load after challenge (as an assessment of activity)(See, *e.g.*, Jin *et al.*, 2000, J Virol. 74:74-82). A reduced level of replication relative to wild-type virus is recognized as an indication of attenuation. Using these animal models, attenuation of the vaccine candidates could be "ranked" relative to wild-type. Although seronegative human subjects are known to be more permissive for RSV-infection, the ranking in these animal models is predictive of the relative ranking of attenuation of the vaccine strains in human subjects (see, *e.g.*, Karron et al., 1997, J. Inf. Diseases 174:1428-36).

8. While formalin-inactivated vaccine approaches for treating RSV were abandoned, subunit and live attenuated vaccines were pursued using these animal models to screen and identify vaccine candidates suitable for human clinical studies. The clinical studies of live attenuated vaccines in human subjects were conducted using iterative "step-down" trials; in other words, the vaccines were evaluated sequentially in adults, seropositive

children, seronegative children, and then infants (see, *e.g.*, Karron, 2005, J Infect Dis. 191:1093-1104).

9. By the mid-1990s, the live attenuated viruses were regarded as the most attractive vaccine candidates. Based on previous experience with live attenuated influenza vaccine, cold passaged (*cp*) and temperature sensitive (*ts*) mutants of RSV were developed. These mutant viruses are unable to replicate at high temperatures, *i.e.*, at core body temperature. As a result, replication *in vivo* is permissive at cooler temperatures in the upper respiratory tract (*i.e.*, an advantage for intranasally administered vaccines), yet restricted at core body temperature in the lower respiratory tract. Several live attenuated RSV vaccines, *e.g.*, *cpts* 248/955; *cpts* 530/1009; and *cpts* 248/404, were generated by chemically inducing additional mutations into cold-passaged mutant RSV (reviewed in Polack & Karron, 2004, Pediatr Infect Dis J. 23:S65-73). These were tested in animal models and entered human clinical trials. The *cpts*-248/404 vaccine was broadly immunogenic in children, and the vaccine recipients showed a reduced rate of viral replication when re-vaccinated. Notably, vaccine recipients in the 6 to 24 month age group had reduced RSV disease on subsequent exposure to wild type RSV. Moreover, none of the recipients developed enhanced disease that was encountered in the formalin-inactivated vaccine trial. Only mild-to-moderate upper respiratory tract congestion, in other words, a stuffy nose, was observed in the youngest infants (reviewed in Wright *et al.*, 2000, J Infect Dis. 182:1331-1342; "Wright 2000"). While this vaccine met the criteria for an RSV vaccine, a commercial product was not developed.

10. In the early to mid-1990s, technical hurdles hampered the development of live attenuated RSV vaccines as commercial products. RSV is a member of the paramyxovirus family.

These viruses have a nonsegmented negative-stranded RNA genome. Mutant viruses of this group could not be generated from plasmid DNA. Instead, live attenuated viruses were generated by random mutagenesis techniques, such as cold passaging and chemical mutagenesis. The genetic stability of the resulting mutant viruses could not be ensured. For example, point mutations resulting from random mutagenesis could mutate back to the wild type nucleotide sequence, thereby reverting the attenuated phenotype to a less attenuated phenotype. Although the RSV vaccines with multiple attenuating mutations typically did not revert completely to a wild type phenotype, genetic instability of the RSV vaccines that were generated using random mutagenesis still hampered the development of a commercial product.

THE TEACHINGS OF THE '388 APPLICATION

11. The '388 application describes a method for the generation of live RSV, and other paramyxoviruses, from plasmid DNA, and the use of this technique to engineer combinations of attenuating mutations into the viral genome for the rational design of viral mutants, including vaccines. Temperature-sensitive mutations known to be attenuating in human subjects could now be built into the virus, accompanied by additional mutations that prevent reversion of the attenuated phenotype. For example, stabilized mutations could now be made by introducing two or three point mutations in a single codon to engineer an amino acid exchange. The likelihood of such stabilized codons to revert to the wild type sequence is reduced compared to the single point mutations that were generated using the old-fashioned random mutagenesis approaches. Alternatively, entire codons could be deleted making reversion of the mutation highly

unlikely -- the mutated virus cannot regain the lost genetic material through homologous recombination because nonsegmented negative strand RNA viruses do not undergo recombination (Collins *et al.*, 2001, In: Chapter 45: Respiratory Syncytial Virus, Fields Virology, 4th Ed., Lippincott Williams & Wilkins).

12. A number of strategies are taught in the '388 application for engineering viral mutants that are infectious and replication competent, including attenuated mutants. In one approach taught in the '388 application, the relative expression levels of individual viral proteins could be changed to alter the replication and/or maturation of progeny virions. According to the '388 application, this can be accomplished by "gene shuffling" (see Section 6.2.1 of the '388 application, in particular p. 39, *ll.* 30-33). Viral genes located at the 3′ end of the genome are expressed at higher levels than those located at the 5′ end. In order to take advantage of this 3′ to 5′ gradient, the '388 application teaches that a viral gene can be translocated within the viral genome to alter its expression level. Another way to change expression levels taught in the '388 application involves altering the non-coding regulatory regions of the genome to down-regulate the expression of any viral gene (see p. 22, *ll.* 3-7 of the '388 application).

13. The specification further teaches that insertions, deletions, and substitutions of individual nucleotides, one or more codons, or entire open reading frames can be introduced into the viral genome (see Section 5.4 of the '388 application, in particular p. 21, *ll.* 29-33, and p. 23, *ll.* 6-10). These genetic modifications can be engineered into the genes of the viral replication apparatus, *i.e.*, the N, P, and L genes, so that replication of the viral genome is reduced (p. 21, *ll.* 1-5; and p. 24, *ll.* 9-15 and 22-27). Alternatively, such modifications of other viral genes, such as F, G, M, and SH, can be engineered to alter viral infectivity

and/or slow virus maturation (p. 21, *ll.* 14-20; and p. 40, *ll.* 21-32). Mutations that reduce replication of the genome and/or slow virus maturation are attenuating because they reduce the generation of progeny virions.

14. While such mutations can be made randomly, the '388 application provides guidance for the rational design of mutations that will affect viral protein function. For example, it describes the use of scanning mutagenesis to remove or disrupt clusters of charged residues without grossly changing the structure of the viral protein, so that protein-protein as well as protein-substrate interactions are altered (see p. 55, *ll.* 1-36, of the '388 application). Cysteine scanning mutagenesis can be used to alter the tertiary structure and activity of viral proteins (see p. 56, *ll.* 8-24, of the '388 application).

15. Furthermore, the '388 application describes assays that can be used to evaluate the impact of the engineered mutation. A minigenome replication system is described to evaluate the functionality of the altered viral proteins, as well as a plaque assay to confirm recovery of infectious, replication competent viral mutants (p. 57, *ll.* 4-18; and p. 59, *ll.* 7-8).

16. The working examples demonstrate the successful use of these engineering strategies to rationally design infectious, replication competent viral mutants. These examples include deletions of the M2-2 and SH genes (Section 10 of the '388 application), insertions and substitutions of the F and G genes (Section 8 of the '388 application), and the removal of charges from the L protein (Section 9 of the '388 application). Infectious replicating viruses carrying these mutations were shown to have an attenuated phenotype as measured by reduced viral replication (p. 59, *ll.* 1-10).

17. The M2-2 deletion mutants made in the working examples were later shown to be attenuated in chimpanzees, rodents, and African Green Monkeys relative to wild type and, in the case of chimpanzees, also relative to *cpts* 248/404 (Jin, 2000, J Virol. 74:74-82; and Jin, 2003, Vaccine 21:3647-3652). The consistency of the attenuation among the different mammalian animal models again verifies that data relating to viral attenuation have predictive value for the degree of attenuation in other mammals.

18. Likewise, the SH deletion mutant made in the working examples was shown to be attenuated relative to wild type RSV in chimpanzees (Jin, 2000, Virology 273:210-218). Further, a combination of *cpts* 248/404 with a deletion of the SH gene, termed *cpts*248/404ΔSH, is attenuated relative to wild type in chimpanzees as well as in seronegative children (Whitehead, 1999, J Virol. 73:3438-3442; and Karron, 2005, J Infect Dis. 191:1093-1104). This observation again affirms that the properties of an RSV mutant in an animal model system are predictive of its properties in humans. Although the deletion of the SH gene did not further attenuate the *cpts* 248/404 mutant, the additional SH deletion is expected to enhance genetic stability and reduce the reversion rate of *cpts* 248/404.

19. Further, the G gene of an RSV B strain was inserted into an intergenic region of an RSV A strain in the working examples of the '388 application (Section 8.2; Figure 9). This chimeric virus, rRSV A2 (B-G), was successfully rescued and shown to be attenuated in cell culture (Figure 9). This working example demonstrates that additional genes can be inserted into an intergenic region of RSV without destroying the viability of the virus, and that insertion of genes into the viral genome is an additional way of reducing viral replication levels. The working examples further show that the open reading frames of

the F and G genes of an RSV A strain can be substituted with those of an RSV B strain (Section 8.1). This virus, rA-G_BF_B, was also successfully rescued demonstrating that substitutions of entire open reading frames can be performed. rA-G_BF_B, has since been shown to be attenuated in cotton rats and African green monkeys relative to wildtype RSV A and RSV B (Cheng, 2001, Virology 283:59-68).

20. I was asked to address a technical issue that was raised by the examiner regarding L mutants; namely, whether infectious replicating virus can be generated from mutants with deletions in the L gene. This appears to be the case. First, the '388 application shows the recovery of infectious, replication competent viruses carrying mutations in the L gene that disrupt or remove charges from the L protein. Moreover, it was known at the time that portions of the L protein containing clustered charges could be deleted without eliminating L protein function. For example, truncated and deleted forms of the L proteins maintained the ability to bind to the P protein (Parks, 1994, J Virol. 68:4862-4872) which is required for viral replication. Further, it has since been shown that replication competent HPIV2, a paramyxovirus, can be constructed with deletions in the L protein (Nolan, 2005, Vaccine 23:4765-4774).

21. The principles taught in the '388 application are not limited to its working examples. Despite minor differences in the exact molecular nature of their M2-2 deletion mutants, different researchers observed similar effects on viral replication resulting from the removal of the M2-2 gene from the viral genome. For example, similar degrees of attenuation were observed for two different M2-2 deletion mutants relative to wild type (Teng, 2000, J Virol.74:9317-9321; Jin, 2000, J Virol. 74:74-82; and Jin, 2003, Vaccine 21:3647-3652). Similarly, two different SH deletion mutants were both found to be

attenuated (Jin, 2000, Virology 273:210-218 and Whitehead, 1999, J Virol. 73:3438-3442). Furthermore, mutations in one paramyxovirus can be transferred to the homologous loci of other paramyxoviruses and are expected to have similar effects (see, *e.g*, Bartlett *et al.*, 2005, Vaccine 23:4631-4646 and Nolan *et al.*, 2005, Vaccine 23:4765-4774). Thus, the guiding principles taught in the '388 application for designing mutations coupled with its working examples for RSV can be applied to other paramyxoviruses.

22. By the mid- to late 1990s, a molecular virologist familiar with the development and testing of *cp* and *ts* RSV mutants that were generated using the old-fashioned random mutagenesis techniques would have known that some of these mutants induced a protective response in children without the development of enhanced disease that was encountered with the formalin-inactivated vaccine, and that appropriate animal models existed to test vaccine candidates for entry into human trials. These virologists would also have been familiar with the sequence information of the mutations that were generated using the random mutagenesis approach and with the structure-function relationship of the genomes of different paramyxoviruses. However, the practitioner would have recognized that genetic instability of the randomly-generated mutants and the inability to make planned mutations into these viral genomes hampered commercial development. The technology and strategies described in the '388 application finally allowed molecular virologists to engineer the viral genomes of paramyxoviruses by stabilizing amino acids known to contribute to attenuated phenotypes, rationally combining mutations into a single viral genome, and testing new mutations in regions of the viral genome that were known to affect viral replication or virus maturation.

Evaluation of the resulting mutants in the established animal model systems was routine. Practitioners in this art were prepared to apply the techniques and strategies taught in the '388 application for generating and developing viral mutants, and have, in fact, done so.

23. Using these teachings, molecular virologists have engineered paramyxoviruses such as RSV, PIV, and hMPV, including vaccine strains (*e.g.*, see Karron *et al.*, 2005, J Infect Dis. 191:1093-1104; "Karron *et al.*"). Karron *et al.* used the recombinant methods described in the '388 application to engineer additional mutations into the genome of *cpts* RSV mutants that had been previously generated by random mutagenesis. These recombinantly produced mutants, rA2*cp*248/404ΔSH and rA2*cp*248/404/1030ΔSH, were shown to be attenuated and immunogenic in adults and children. Recombinant hMPV has been generated from cDNA (Biacchesi, 2004, Virology 321:247-259). A deletion of the SH gene or a deletion of the G gene and the SH gene from the genome of recombinant hMPV resulted in attenuation in hamsters, where the hMPV deletion mutants also induced hMPV-neutralizing antibodies (Biacchesi, 2004, J Virol. 78:12877-12887). Successful application of the methods taught in the '388 application to PIV type 3 ("PIV-3") are for example shown by Pennathur (2003, J. Gen'l. Virol. 84: 3253-326/1; "Pennathur") . In Pennathur, a bovine PIV-3 virus whose F gene was substituted with the F gene of human PIV3 was shown to be attenuated in rhesus monkeys and to protect these animals from human PIV-3 challenge (Pennathur).

24. In view of the foregoing facts and analysis, I have concluded that, in the mid- to late 1990s, a molecular virologist following the teachings and guidance set out in the '388 application would, using ordinary skill, be able to (a) engineer mutant infectious,

replication competent viruses belonging to the paramyxovirus family, in particular, RSV, and (b) generate vaccine strains of these viruses.

25. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

03 OCT 2005
DATE

Richard R. Spaete
RICHARD R. SPAETE

Attachments:

Alphabetical List of References Cited

Copies of References Cited

Exhibit A to Declaration of Richard R. Spaete

current August 2005

CURRICULUM VITAE

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Education and Work Experience

1974	B.S., University of Wisconsin-Eau Claire - Biology/Chemistry
1978	M.S., University of Montana, Missoula, Montana -Microbiology
1982	Ph.D., University of Chicago - Committee on Virology
12/1/82 to 3/31/83	Postdoctoral Fellow, University of Chicago
3/31/83 to 10/31/86	Postdoctoral Fellow, Stanford University
11/1/86 to 6/1/88	Scientist, Chiron Corporation, Emeryville, CA
6/1/88 to 2/1/92	Principal Scientist, Chiron Corporation, Emeryville, CA
2/1/92 to 10/1/92	Senior Scientist, Chiron Corporation, Emeryville, CA
10/1/92 to 7/22/94	Senior Scientist, Aviron, Burlingame, CA
7/22/94 to 9/1/02	Director, Aviron/MedImmune Vaccines, Mountain View, CA
9/1/02 to Present	Senior Director, MedImmune Vaccines, Inc. Mtn. View, CA

Academic and Professional Honors and Activities

10/1/78 - 9/30/82	PHS Predoctoral Traineeship 5T32CA09241-05

12/1/82 - 10/31/83	PHS Postdoctoral Traineeship 5T32CA09241
6/3/83 - 5/31/84	Dean's Fellowship, Stanford University
6/1/84 - 6/1/85	Leukemia Research Foundation Postdoctoral Award
1985	Invited reviewer, Science
6/1/85 - 6/1/86	Leukemia Research Foundation Postdoctoral Award
5/1/90 - 10/31/90	SBIR Phase I Grant 1 R43 AI29273-01
1990	Invited reviewer, Virology
9/3/90 - 5/31/91	SBIR Phase I Grant 1 R43 AI29790-01
1993	Invited reviewer, The Journal of Infectious Diseases
1994-05	Invited reviewer, Archives of Virology, The Journal of Infectious Diseases, Antiviral Research, Journal of Virology, Vaccine, Virology
2004	Invited scientist reviewer – 2004 Grand Challenges in Global Health
2005-07	Editorial Board, Journal of Virology

Management Courses

1994	Project Management in the Research-Based Pharmaceutical Industry sponsored by PRMA's Project Management and Finance Section
1994	R&D and Engineering Management sponsored by the American Management Association

PUBLICATIONS

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American Association for the Advancement of Science
American Society of Microbiology
American Society for Virology
International Society for Vaccines
Society for General Microbiology

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Human parainfluenza virus type I (HPIV1) vaccine candidates designed by reverse genetics are attenuated and efficacious in African green monkeys

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Abstract

A set of recombinant, live attenuated human parainfluenza virus type 1 (rHPIV1) vaccine candidates was evaluated for attenuation, immunogenicity, and protective efficacy in African green monkeys (AGMs). Temperature sensitive (*ts*) and non-*ts* attenuating (*att*) mutations in the P/C and L genes were introduced individually or in various combinations into rHPIV1, including the C^{R84G} and HN^{T353A} mutations identified in the present work and the C^{F170S}, L^{Y942A}, and L^{L992C} mutations identified previously. The rHPIV1 vaccine candidates exhibited a spectrum of attenuation in AGMs. One genetically and phenotypically stable vaccine candidate, $rC^{R84G/F170S}L^{Y942A/L992C}$, was attenuated and efficacious in AGMs and is a promising live attenuated intranasal HPIV1 vaccine candidate suitable for clinical evaluation.
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1. Introduction

Human parainfluenza virus type 1 (HPIV1) is a significant cause of severe respiratory tract disease in infants and young children [1]. HPIV1 is an enveloped, non-segmented, single-stranded, negative-sense RNA virus belonging to the subfamily *Paramyxovirinae* within the *Paramyxoviridae* family, which also includes the HPIV2 and HPIV3 serotypes. These serotypes can be further classified as belonging to either the *Respirovirus* (HPIV1 and HPIV3) or *Rubulavirus* (HPIV2) genus and are immunologically distinct in that primary infection does not result in cross-neutralization or cross-protection [2,3]. The HPIV1 genome encodes three nucleocapsid-associated proteins including the nucleocapsid protein (N), the phosphoprotein (P) and the large polymerase (L) and three envelope-associated proteins including the internal matrix protein (M) and the fusion (F) and hemagglutinin-neuraminidase (HN) transmembrane surface glycoproteins. F and HN are the two viral neutralization antigens and are the major viral protective antigens [4]. In addition, the P/C gene of HPIV1 contains a second open reading frame that encodes four accessory C proteins, C′, C, Y1 and Y2, that initiate at four separate translational start codons and are carboxy co-terminal (Fig. 1). However, it is unclear whether the Y2 protein is actually expressed in HPIV1 infection [5]. The C proteins of murine PIV1 (MPIV1, Sendai virus) inhibit virus replication and transcription and also play an important role in evasion of the host's innate immune response by interrupting both the production of interferon and signaling through its receptor [6–11].

The HPIVs cause respiratory tract disease ranging from mild illness, including rhinitis, pharyngitis, and otitis media, to severe disease, including croup, bronchiolitis, and pneumonia [1,12–16]. HPIV1, HPIV2 and HPIV3 have been identified as the etiologic agents responsible for 6.0%, 3.3%

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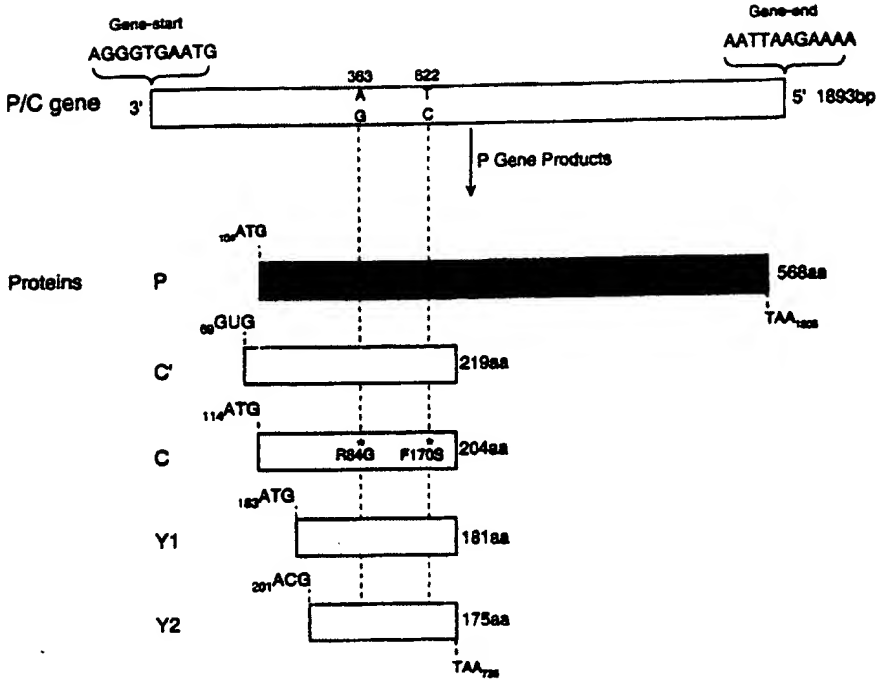

Fig. 1. Schematic representation of the nucleotide substitutions introduced into the P/C gene of HPIV1 and the accompanying amino acid changes in the encoded proteins. The P protein is denoted by a dark gray rectangle and the C accessory proteins (C', C, Y1, and Y2), which are encoded by the +1 reading frame relative to P, are shown in light gray. Nucleotide substitutions within the P/C gene (numbered according to the sequence of the P/C gene) are shown with the wild type assignment on top and the mutant assignment underneath. The nucleotide positions for the start codons of the $C'_{(69)}$, $P_{(104)}$, $C_{(114)}$, $Y1_{(183)}$ and $Y2_{(201)}$ ORFs are indicated, as is the position of the stop codon for the C accessory proteins (TAA_{726}) and the P protein (TAA_{1808}). Mutation nomenclature includes wild type amino acid, codon position and codon change, as referred to throughout the paper. P/C mutations are named according to the corresponding amino acid substitution and sequence position in the C protein (note that the same substitutions would be present in the C', Y1 and Y2 proteins, but the amino acid numbering differs due to the different lengths at the amino termini, and hence is shown only for C). The nucleotide substitution giving rise to the R84G mutation in C also results in a substitution in P (E87G), whereas the F170S mutation in C is silent in P.

and 11.5%, respectively, of hospitalizations of infants and young children for respiratory tract disease [1,17]. Together these viruses account for approximately 20% of all pediatric hospitalizations due to respiratory disease. HPIVs are also recognized as causes of respiratory disease in adults and immunocompromised patients [18–23]. A licensed vaccine is currently not available for any of the HPIVs.

Our laboratory is developing live attenuated intranasal virus vaccines for a variety of respiratory tract pathogens including influenza A and B viruses, respiratory syncytial virus, and the parainfluenza viruses [24–26]. The advantages of a live attenuated virus vaccine include the ability of such vaccines to: (i) induce the full spectrum of protective immune responses including serum and local antibodies and CD4+ and CD8+ T cells [27]; (ii) infect and immunize in the presence of maternal antibody permitting use in young infants [25,26]; (iii) cause an acute, self-limited infection which is readily eliminated from the respiratory tract; and (iv) replicate to high titers in acceptable substrates including the allantoic cavity of eggs for influenza viruses or Vero cells for RSV and HPIV vaccines making these vaccines commercially feasible. In fact, the recent licensure of the trivalent live attenuated influenza A virus vaccine (Flumist™) indicates that it is possible to achieve an acceptable balance between attenuation and immunogenicity with a live attenuated respiratory virus vaccine [28].

The licensed cold-adapted influenza A viruses as well as a promising HPIV3 vaccine candidate contain both temperature sensitive (*ts*) and non-*ts* attenuating (*att*) mutations, which are known to act in concert to enhance the phenotypic stability of the live attenuated respiratory virus vaccines [24,29,30]. The development of a reverse genetics system for HPIV1 provides the capability to generate live attenuated recombinant HPIV1 (rHPIV1) vaccine candidates by the introduction of one or more *ts* and non-*ts att* mutations into wild type HPIV1 [4,31]. Since respiratory viruses with mutations in proteins with anti-interferon activities are attenuated in vivo, the C accessory proteins are prime targets for inactivation by mutation [32,33]. In addition, mutations in L have been identified that attenuate respiratory viruses for rodents or primates [34,35].

Attenuating mutations identified in the L genes of RSV and HPIV3 and the C genes of MPIV1 and HPIV3 were previously transferred to the homologous loci of HPIV1 identified by sequence alignments to generate live attenuated HPIV1 vaccine candidates [31,36–40]. Specifically, amino acid substitutions introduced individually at position 170 in the C protein of HPIV1 [31] and at positions 456, 942, 992 and 1558

in L [31,38–40] attenuated HPIV1 for replication in the respiratory tract of hamsters. The mutation at position 170 in C specified a non-*ts att* phenotype whereas those in L specified either a *ts* or non-*ts att* phenotype [31,40]. The combination of L gene mutations rendered viruses more *ts* and more attenuated in hamsters than either mutation alone [31]. The codons at positions 942 and 992 were systematically mutated to achieve enhanced phenotypic stability and increased attenuation [40]. At position 942, the original rL^{Y942H} virus was mutated to generate rL^{Y942A} [40], a virus that possessed a similar level of temperature sensitivity and attenuation as rL^{Y942H} but that would require three nucleotide substitutions in the Y942A codon to generate a codon that specified a att^+ phenotype [40]. The rL^{Y942A} mutant was confirmed to exhibit increased genetic and phenotypic stability over that of rL^{Y942H} [40]. Similarly, a 2-nucleotide substitution at position 992 (Leu to Cys) was found to specify the highest level of temperature sensitivity and attenuation among recombinants with a change at codon 992 [40].

In the present study, these previous observations were extended in several respects. First, rHPIV1 vaccine candidates were generated to contain new combinations of mutations that included the stabilized codon at 942 and the partially-stabilized codon at 992 in L. Second, a pair of novel spontaneous mutations in C (R84G) and HN (T553A) was identified, characterized, and used to generate novel vaccine candidates. Third, new rHPIV1 combination vaccine candidates were evaluated in hamsters. Fourth, the rHPIV1 vaccine candidates were evaluated in African green monkeys (AGMs), whose anatomical and phylogenetic relatedness to humans makes them suitable as the penultimate step prior to clinical trials. rHPIV1 vaccine candidates were identified that exhibited a spectrum in their level of attenuation, immunogenicity, and efficacy in hamsters or AGMs. Several of the rHPIV1 vaccine candidates appeared to have achieved an acceptable balance between attenuation and immunogenicity for AGMs and thus represent promising vaccine candidates for use in humans.

2. Materials and methods

2.1. Cells and viruses

LLC-MK2 cells (ATCC CCL 7.1) and HEp-2 cells (ATCC CCL 23) were maintained in Opti-MEM I (Gibco-Invitrogen Inc., Grand Island, NY) supplemented with 5% FBS, gentamicin sulfate (50 μg/ml), and 2mM glutamine (Gibco-Invitrogen Inc.). Wild type biologically-derived HPIV1 Washington/20993/1964 was isolated previously from a clinical sample and passaged in fetal rhesus lung (FRhL) cells. Previously, this virus was (1) subjected to one additional passage on LLC-MK2 cells and designated $HPIV1_{LLC1}$ or (2) subjected to four additional passages on LLC-MK2 cells and designated $HPIV1_{LLC4}$ [41]. $HPIV1_{LLC1}$, $HPIV1_{LLC4}$, and the rHPIV1 mutants were grown in LLC-MK2 cells in the presence of 5 μg/ml added trypsin (BioWhittaker, Walkerville, MD) as described previously [4].

2.2. Construction of mutant HPIV1 cDNA

Mutations were introduced into the appropriate rHPIV1 subgenomic clones [31] using the Advantage-HF PCR Kit (Clontech Laboratories, Palo Alto, CA) with a modified PCR mutagenesis protocol described elsewhere [42]. The entire PCR amplified subgenomic clone was sequenced using a Perkin-Elmer ABI 3730 sequencer with the Big Dye sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, UK) to confirm that the subclone contained the introduced mutation but did not contain any adventitious mutations introduced during PCR amplification. Full-length HPIV1 antigenomic cDNA clones (FLCs) containing the mutations were assembled using standard molecular cloning techniques [4], and the region containing the introduced mutation in each FLC was sequenced as described above to ensure that the FLC contained the introduced mutation.

2.3. Recovery of rHPIV1 mutant viruses

Recovery of rHPIV1 mutants was performed as described previously using support plasmids containing the appropriate mutations [4]. To confirm that the recovered rHPIV1 mutants contained the appropriate mutations and lacked adventitious mutations in the P and/or L genes, viral RNA (vRNA) was isolated from infected cell supernatant fluids using the Qiaquick vRNA kit (Qiagen Inc., Valencia, CA) and was reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen Inc., Carlsbad, CA) and amplified using the Advantage cDNA PCR Kit (Clontech Laboratories). The P or L genes were sequenced in their entireties from uncloned RT-PCR product. The recovered rHPIV1 viruses were cloned by two successive rounds of terminal dilution using LLC-MK2 monolayers in 96-well plates (Costar, Corning Inc., Corning, NY). The presence of the introduced mutations in the biologically cloned rHPIV1 viruses was confirmed by sequence analysis of vRNA using RT-PCR as described above.

2.4. Characterization of rHPIV1 vaccine candidates

The *ts* phenotype for each of the mutant rHPIV1 viruses was determined by comparing its level of replication to that of $HPIV1_{LLC1}$ at 32 °C and at 1 °C increments from 35 °C to 40 °C, as described previously [43]. Briefly, each virus was serially diluted 10-fold in 96-well LLC-MK2 monolayer cultures in L-15 media (Gibco-Invitrogen Inc.) containing trypsin and antibiotics with four replicate wells per plate. Replicate plates were incubated at the temperatures indicated above for seven days, and virus infected cultures were detected by hemadsorption with guinea pig erythrocytes. Virus titer at each temperature was determined in three to six separate experiments and is expressed as the mean $\log_{10}$ 50 percent tissue culture infectious dose per milliliter

($TCID_{50}$/ml). The mean titer at an elevated temperature was compared to the mean titer at 32 °C, and the reduction in mean titer was determined. The shut-off temperature of a rHPIV1 mutant is defined as the lowest temperature at which the reduction in virus titer compared to its titer at 32 °C was 100-fold greater than the difference of $HPIV1_{LLC1}$ between the same two temperatures.

2.5. Evaluation of replication of viruses in hamsters

Four-week-old Golden Syrian hamsters in groups of 5 or 6 per virus were inoculated intranasally (i.n.) with 0.1 ml L-15 containing 10^6 $TCID_{50}$ of $HPIV1_{LLC1}$ or rHPIV1. Four days later, the nasal turbinates and lungs were collected as previously described [4]. Virus present in the tissue homogenates was quantified by titration on LLC-MK2 monolayers at 32 °C. Infected cells were detected on day seven post-infection by hemadsorption with guinea pig erythrocytes. The mean titer (log_{10} $TCID_{50}$/g) was calculated for each group of six hamsters. The limit of detection was 1.5 log_{10} $TCID_{50}$/g.

2.6. Evaluation of replication of viruses in African green monkeys and efficacy against challenge

AGMs in groups of two to four animals were inoculated i.n. and intratracheally (i.t.) with 10^6 $TCID_{50}$ of either $HPIV1_{LLC1}$ or a rHPIV1 vaccine candidate in a 1 ml inoculum at each site. Nasopharyngeal (NP) swab samples were collected daily from day 0–10 post-inoculation, and tracheal lavage (TL) samples were collected on days 2, 4, 6, 8, and 10 post-inoculation. The specimens were flash frozen and stored at −80 °C until all specimens had been collected. Virus present in the samples was titered on LLC-MK2 cell monolayers in 96-well plates and in 24-well plates that were incubated at 32 °C for 7 days. Virus was detected by hemadsorption, and the mean log_{10} $TCID_{50}$/ml was calculated for each sample day. The limit of detection was 0.5 log_{10} $TCID_{50}$/ml. The mean of the total virus shed was determined from the sum, calculated for each animal individually, of virus titer on each day of sampling. The lower limit of detection is 5.5 log_{10} $TCID_{50}$/ml for NP swabs and 2.5 log_{10} $TCID_{50}$/ml for TL samples.

On day 28 post-inoculation, the AGMs were challenged i.n. and i.t. with 10^6 $TCID_{50}$ of $HPIV1_{LLC1}$ in 1 ml at each site. NP swab samples were collected for virus quantitation on days 0, 2, 4, 6 and 8 post-challenge, and TL samples were collected on days 2, 4, 6 and 8 post-challenge.

2.7. Evaluation of immune responses in African green monkeys

Serum was collected from each monkey on days 0 and 28 post-immunization and day 28 post-challenge (day 56 post-first inoculation), and HPIV1 neutralizing and/or hemagglutination inhibiting (HAI) antibody titers were determined. The level of serum HPIV1 neutralizing antibodies was determined using a complement-enhanced assay. Two-fold serial dilutions of AGM serum were combined with an equal volume of virus suspension containing approximately $10^{4.9}$ $TCID_{50}$ of rHPIV1 modified to express green fluorescent protein (rHPIV1-GFP) that served as a novel indicator virus. This modified rHPIV1 expressed the GFP as an additional gene unit inserted upstream of the N gene and was generated as previously described for rHPIV1 vaccine candidates [4,44]. The rHPIV1-GFP expresses GFP in infected cells, facilitating the detection of infected cultures without hemadsorption or immunostaining. The virus and serum mixture was incubated at 37 °C for 1 h in MEM (Gibco-Invitrogen Inc.) and 10% guinea pig complement (Cambrex Inc., Walkerville, MD) and was transferred to LLC-MK2 monolayers in 96-well plates. After a 1 h adsorption period, the monolayers were washed three times to remove residual serum and complement, and Opti-MEM I containing trypsin was added. Infected monolayer cultures were incubated at 32 °C for 7 days, and read for GFP expression on the Typhoon phosphorimager using a Typhoon 8600 scanner (Molecular Dynamics Inc., Sunnyvale, CA) control program (settings: fluorescence; filter, 526-SP green fluorescein). The end-point was defined as the highest dilution of the serum/virus mixture that exhibited a 50% reduction in the level of GFP expression. The neutralization titer is the highest dilution of serum at which 50% of the wells exhibited this level of reduction in GFP expression, determined using the IPLab Gel programme (Signal Analytics Corp., Vienna, VA).

HAI antibody titers to HPIV1 were determined at 21 °C as described previously [45] by using 0.5% guinea pig erythrocytes and $HPIV1_{LLC1}$ as the antigen. The antibody titer was defined as the end-point serum dilution that inhibited hemagglutination and is expressed as the mean reciprocal log_2 ± standard error (S.E.).

2.8. Statistical analysis

The Prism 4 (GraphPad Software Inc., San Diego, CA) one-way ANOVA test (Student–Newman–Keuls multiple comparison test) was used to assess statistically significant differences between data groups ($P < 0.05$).

3. Results

3.1. Identification of spontaneous mutations in the P/C and HN genes of HPIV1

The HPIV1 Wash/64 clinical isolate was passaged previously on FRhL cells and confirmed to be virulent in adult volunteers [41]. This FRhL cell grown preparation was serially passaged four times at 32 °C on LLC-MK2 cells to generate the uncloned virus preparation, $HPIV1_{LLC4}$, which was used to determine the original HPIV1 consensus sequence [4]. Previously, we generated a recombinant HPIV1 based on the genome consensus sequence of

$HPIV1_{LLC4}$, designated $rHPIV1_{LLC4}$. In the present study, $rHPIV1_{LLC4}$ and the biologically-derived $HPIV1_{LLC1}$ wild type virus, passaged only once in LLC-MK2 cells, were used to infect AGMs. We noted a nearly two log_{10} decrease in the level of virus shedding in the upper respiratory tract (URT) of monkeys infected with $rHPIV1_{LLC4}$ compared to $HPIV1_{LLC1}$ (Table 1). This apparent *att* phenotype for $rHPIV1_{LLC4}$ was unexpected since, with the exception of two silent nucleotide substitutions in the L polymerase ORF (T to C changes at nucleotides 10706 and 14267), the $rHPIV1_{LLC4}$ sequence was identical to that determined for its biologically-derived counterpart, $HPIV1_{LLC4}$.

It was possible that passaging the wild type HPIV1 four times on LLC-MK2 cells resulted in the acquisition of one or more attenuating point mutations that could be responsible for the *att* phenotype observed in AGMs. In order to address this, we determined the complete genome consensus sequences of the $HPIV1_{LLC1}$ and $HPIV1_{LLC4}$ preparations. The sequences were found to differ at several positions within the coding regions, three of which are translationally silent (HPIV1 nucleotides 1097 C to T, 2815 A to G, 4625 A to C, with the nucleotide positions and assignments according to the complete antigenome sequence) and two of which result in amino acid substitutions. The translationally silent mutations in the coding regions were presumed not to contribute to the *att* phenotype in AGMs. One of the two amino acid coding changes was in the P/C gene at nucleotide 2103 (A to G) and encodes a mutation in the C protein (Arg-84 to Gly) and an accompanying mutation in P (Glu-87 to Gly, Fig. 1). These P/C mutations are collectively termed C^{R84G} (Fig. 1). The second coding change occurred in the HN gene at nucleotide 8559 (A to G) and encodes a mutation in the HN protein (Thr-553 to Ala, termed HN^{T553A}) (Table 2). Thus, our previous analysis of rHPIV1 and mutant derivatives [31,40] was performed in the context of the previously unrecognized C^{R84G} and HN^{T553A} amino acid substitutions that had been acquired by $HPIV1_{LLC4}$ during passage in LLC-MK2 cells and which had been faithfully copied into its recombinant version, $rHPIV1_{LLC4}$.

It was presumed that either or both of the spontaneous P/C or HN gene mutations specified the *att* phenotype observed in AGMs. Sequence alignments between HPIV1 and various paramyxoviruses including MPIV1, HPIV3 and BPIV3 showed that the amino acid assignment at position 553 in the HN gene is not highly conserved. Furthermore, the HN^{T553A} substitution has also been found in another wild type strain of HPIV1, the Mil-48/91 isolate of HPIV1 (accession number: AAB17171) [46]. Therefore, we anticipated that HN^{T553A} did not specify the *att* phenotype. In contrast, sequence alignments as described above revealed a high degree of conservation at position 84 in C (and position 87 in P), suggesting that the C^{R84G} mutation was more likely to be responsible for the attenuation of $rHPIV1_{LLC4}$ (rC^{R84G}) in AGMs. To investigate this, we constructed two recombinant HPIVs, one designated rHN^{T553A}, which contained the HN^{T553A} mutation but lacked the C^{R84G} mutation, and one designated rC^{R84G*}, which contained the C^{R84G} mutation but lacked the HN^{T553A} mutation. Their replication was evaluated in AGMs (Table 1). Both rHN^{T553A} and rC^{R84G} replicated to the level of wild type $HPIV1_{LLC1}$ in the URT of AGMs, indicating that the HN^{T553A} and C^{R84G} mutations are both required to attenuate $rHPIV1_{LLC4}$ in AGMs.

Hereafter in this article, viruses will be named according to the amino acid substitutions that they possess compared to $HPIV1_{LLC1}$, a nomenclature designed to facilitate virus-to-virus comparison. However, since all viruses except rC^{R84G*}

Table 1
The spontaneous C^{R84G} and HN^{T553A} mutations in $HPIV1_{LLC4}$ and $rHPIV1_{LLC4}$ together specify an attenuation phenotype in the upper respiratory tract of African green monkeys

Virus[a]	No. of animals	Mean peak virus titer (log_{10} $TCID_{50}$/ml)[b]		Mean sum of the daily virus titers (log_{10} $TCID_{50}$/ml)[c]	
		NP swab[d]	TL[e]	NP swab	TL
$HPIV1_{LLC1}$	8	4.0 ± 0.2	4.3 ± 0.4	25.3 ± 1.7	14.3 ± 1.9
rHN^{T553A}	4	4.3 ± 0.2	4.6 ± 0.2	25.4 ± 0.7	13.7 ± 1.8
rC^{R84G}[f]	4	3.6 ± 0.4	4.1 ± 0.5	21.5 ± 1.7	11.7 ± 2.5
$rHPIV1_{LLC4}$ (rC^{R84G})[g]	12	2.1 ± 0.2[h]	4.8 ± 0.3	11.0 ± 0.9[h]	14.3 ± 1.1

[a] Monkeys were inoculated i.n. and i.t. with 10^6 $TCID_{50}$ of the indicated virus with a 1 ml inoculum at each site. Data are representative of one to four experiments. $rHPIV1_{LLC4}$ is a recombinant version of virus that was subjected to three further passages on LLC-MK2 cells compared to $HPIV1_{LLC1}$, and contains two amino acid substitutions, namely C^{R84G} and HN^{T553A} (see the text). rC^{R84G} and rHN^{T553A} are identical to $HPIV1_{LLC4}$ except for that, of these two amino acid substitutions, they contain only the indicated one.

[b] Virus titrations were performed on LLC-MK2 cells at 32 °C. Mean of the peak virus titers ± S.E. for the animals in each group. The limit of detection was 0.5 log_{10} $TCID_{50}$/ml.

[c] Mean sum of the daily virus titers: the sum of the titers for all of the days of sampling was determined for each animal individually, and the mean was calculated for each group. The lower limit of detection is 5.5 log_{10} $TCID_{50}$/ml for NP swabs and 2.5 log_{10} $TCID_{50}$/ml for TL samples.

[d] NP swab samples were collected on days 0–10 post-infection.

[e] TL samples were collected on days 2, 4, 6, 8, and 10 post-infection.

[f] Indicates that this rHPIV1 does not contain the HN^{T553A} mutation, all other recombinant viruses containing the C^{R84G} mutation also contain the HN^{T553A} mutation (see the text).

[g] $rHPIV1_{LLC4}$ is hereafter referred to as rC^{R84G}, indicating that it contains the *att* combination of C^{R84G} and HN^{T553A} mutations (see the text).

[h] Statistically significant reduction compared to $HPIV1_{LLC1}$ titer, $P < 0.001$ (Student–Newman–Keuls multiple comparison test).

Table 2
Summary of the mutations introduced into the rHPIV1 genome

Protein	Mutation nomenclature[a]	Codon position	Amino acid change (wild type → mutant)	Codon change (wild type → mutant[b])	No. nucleotide changes for reversion to the wild type amino acid
C	C^{R84G}[c]	84	R → G	AGA → GGA	1
P	C^{R84G}[c]	87	E → G	GAG → GGG	1
C	C^{F170S}[c]	170	F → S	TTC → TCC	1
HN	HN^{T553A}	553	T → A	ACC → GCC	1
L	L^{F456L}	456	F → L	TTT → CTG	2
L	L^{Y942H}	942	Y → H	TAT → CAC	1[d]
L	L^{Y942A}	942	Y → A	TAT → GCG	3
L	L^{L992F}	992	L → F	TTA → TTT	1
L	L^{L992C}	992	L → C	TTA → TGC	2
L	L^{L1558I}	1558	L → I	TTT → CTG	2

[a] The nomenclature used to describe each mutation identifies the protein in regular script and provides the wild type amino acid, the codon position, and the mutant amino acid in that order in superscript.
[b] The altered nucleotides are underlined.
[c] The C^{R84G} nucleotide substitution also results in an E87G substitution in the P protein but the C^{F170S} mutation is silent in P (see Fig. 1).
[d] The codon TAC also codes for tyrosine, therefore only one nucleotide change is required for reversion to the wild type assignment.

and rC^{F170S} possess the HN^{T553A} mutation, it will not be included in the names. Thus, $HPIV1_{LLC4}$ will hereafter be referred to as rC^{R84G}, since compared to $HPIV1_{LLC1}$ it contains the C^{R84G} and HN^{T553A} mutations (whereas rC^{R84G*} has only the C^{R84G} mutation).

To examine the possibility that the point mutation encoding the amino acid substitutions in the P/C gene, C^{R84G}, was an adaptation to growth in LLC-MK2 cells, the biologically-derived $HPIV1_{LLC1}$ was sequentially passaged seven times on Vero cell or LLC-MK2 monolayers at 32 °C. The entire P/C gene consensus sequence was determined for each of the two virus preparations. This showed that nucleotide 2103 (A) was unchanged after seven additional passages of $HPIV1_{LLC1}$ in either Vero cells or LLC-MK2 cells. Furthermore, no other coding mutations arose in the P/C gene in these two preparations. This indicates that the nucleotide 2103 A to G change in the P/C gene that arose in $HPIV1_{LLC4}$ does not invariably occur upon passage in LLC-MK2 cells.

3.2. Construction and recovery of mutant rHPIV1 viruses

Previously we had identified several mutations in the C and L proteins of HPIV1 that conferred a moderate to high level of attenuation of replication in the respiratory tract of hamsters, including mutations that specified *ts* and non-*ts att* phenotypes [31,40]. In the present study, novel recombinant HPIV1 viruses were generated that incorporated various sets of the mutations in the P/C or L genes indicated in Table 2 including the stabilized mutations at positions 942 and 992 in L [40]. Each of the mutations in Table 2 has been described previously [31,40] except for C^{R84G} and HN^{T553A}. The C^{F170S} is a point mutation in HPIV1 that corresponds to a homologous *att* mutation in MPIV1 that also arose upon passage of this virus on LLC-MK2 cells [37]. This point mutation codes for an amino acid substitution in the C protein, at position 170 (F to S), and in the C′, Y1, and Y2 proteins but is silent in the P protein (Fig. 1). The F456L, Y942H, Y942A, L992F, L992C and L1558I mutations in L (Table 2) were previously recovered in rHPIV1 and were characterized in vitro and in hamsters [31,40]. The codons at positions 456, 942, 992, and 1558 in the L protein were modified such that two or three nucleotides would be required to revert to the wild type amino acid assignment at those positions. The mutations in Table 2 were introduced into the pFLC HPIV1 antigenomic cDNA individually or in various combinations to yield the panel of rHPIV1 viruses listed in Table 3. These viruses were recovered following transfection in HEp-2 cells and were biologically cloned in LLC-MK2 cells. The purpose in designing a panel of rHPIV1s containing combinations of attenuating mutations was to generate vaccine candidates with a wide spectrum in their level of attenuation and to achieve a greater level of genetic and phenotypic stability.

The kinetics of replication and peak virus titer of a subset of the rHPIV1 candidates were determined in LLC-MK2 cells at 32 °C over a 7-day period (Fig. 2). Although all of the rHPIV1 candidates replicated efficiently, reaching a peak titer of at least 10^7 $TCID_{50}$/ml, there was a modest reduction in the rate of growth and peak virus titer compared to $HPIV1_{LLC1}$ for the combination mutants $rC^{R84G}L^{Y942A/L992C}$ and $rC^{R84G/F170S}L^{Y942A/L992C}$ (1.5 $\log_{10}$ $TCID_{50}$/ml and 1.8 $\log_{10}$ $TCID_{50}$/ml, respectively (Fig. 2)).

3.3. Temperature sensitivity of replication in vitro

The *ts* phenotype of a subset of rHPIV1 candidates bearing individual P/C and L gene mutations was previously examined [31,40]. In the present study the level of temperature sensitivity was determined for the full set of rHPIV1s bearing individual or combined mutations to define the contribution of each mutation to the level of temperature sensitivity of rHPIV1 individually and in combination (Table 3). The rHPIV1 viruses bearing only P/C or HN gene mutations failed to exhibit a *ts* phenotype, however, several of the L gene mutations contributed to a high level of temperature sensitivity. For example, the L protein Y942A and L992C

Table 3
The level of temperature sensitivity of replication of rHPIV1 mutants in vitro[a]

Virus		Virus titer at 32 °C[b]	Mean reduction (log_{10}) in virus titer at indicated temperature[b,c]					
			35 °C	36 °C	37 °C	38 °C	39 °C	*ts*[c]
1	$HPIV1_{LLC1}$	7.9	0.2	0.1	0.2	0.7	1.4	
2	rHN^{T553A}	8.0	−0.4	0.1	0.1	−0.1	1.2	No
3	rC^{R84G*}	9.2	0.4	0.4	0.8	0.3	1.8	No
4	rC^{R84G}	7.8	−0.4	−0.4	−0.3	0.1	0.9	No
5	rC^{F170S}	7.9	−0.1	−0.4	0.5	1.4	1.9	No
6	$rC^{R84G/F170S}$	7.1	0.3	0.0	0.3	0.8	1.2	No
7	$rC^{R84G}L^{Y942Hd,e}$	8.0	–	1.4	**3.1**[c]	5.1	5.8	Yes
8	$rC^{R84G}L^{L992Fd,e}$	8.1	0.2	0.3	1.3	0.9	2.4	No
9	$rC^{R84G}L^{L1558Ie}$	7.8	–	0.4	0.2	2.5	2.4	No
10	$rC^{R84G}L^{F456Le}$	7.1	–	0.7	**2.4**	4.9	≥5.6	Yes
11	$rC^{R84G}L^{Y942A}$	7.4	0.5	1.0	**3.0**	5.0	6.3	Yes
12	$rC^{R84G}L^{L992C}$	7.3	−0.1	0.3	0.9	**2.8**	5.5	Yes
13	$rC^{R84G}L^{Y942A/L992C}$	7.2	1.9	**2.8**	5.7	≥6.3	≥6.3	Yes
14	$rC^{R84G/F170S}L^{Y942A/L992C}$	7.1	**2.3**	4.2	≥5.9	≥5.9	≥5.9	Yes

[a] Data are the mean of three to six experiments.
[b] Viruses were titrated on LLC-MK2 cells at either permissive (32 °C) or potentially restrictive (35 °C–39 °C) temperatures for 7 days. The limit of detection was 1.2 log_{10} $TCID_{50}$/ml.
[c] Values in bold indicate restricted replication, where the mean log_{10} reduction in virus titer at a given temperature vs. 32 °C was 2.0 log_{10} or greater than that of the $HPIV1_{LLC1}$ at the same two temperatures. Underlined values indicate viral shut-off temperature, the lowest temperature at which restricted replication is observed. A virus is designated *ts* if restricted replication at 35 °C–39 °C is observed.
[d] These data have been previously published [39] and is included here for the purposes of comparison.
[e] These data have been previously published [30] and is included here for the purposes of comparison.
[*] Indicates that this rHPIV1 does not contain the HN^{T553A} mutation, all other recombinant viruses containing the C^{R84G} mutation also contain the HN^{T553A} mutation (see the text).

mutations in $rC^{R84G}L^{Y942A}$ and $rC^{R84G}L^{L992C}$, respectively, specified a *ts* phenotype with shut-off temperatures of 37 °C and 38 °C, respectively. Combining the two L protein substitutions specified an even higher level of temperature sensitivity ($rC^{R84G}L^{Y942A/L992C}$, 36 °C shut-off). Surprisingly, the level of temperature sensitivity was further increased when the non-*ts* C^{F170S} mutation was added to this combination, making $rC^{R84G/F170S}L^{Y942A/L992C}$ the vaccine candidate with the highest level of temperature sensitivity (35 °C shut-off). The mechanism for this effect is not known. Thus, both *ts* and non-*ts* mutations were identified, and rHPIV1 vaccine candidates exhibiting a broad range of tempera-

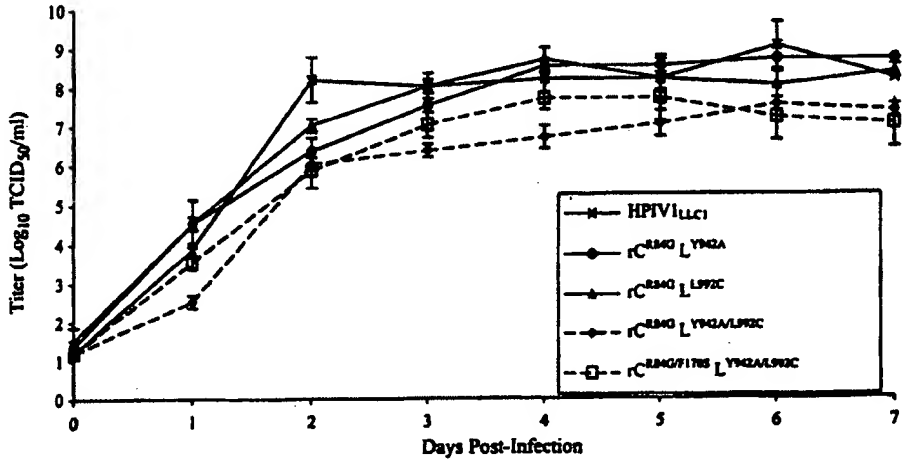

Fig. 2. Comparison of the growth curve of $HPIV1_{LLC1}$ and various rHPIV1 vaccine candidates with the indicated mutations in the P/C and L genes. Monolayers of LLC-MK2 cells were infected at a multiplicity of infection of 0.01, and the cultures were incubated at 32 °C. Aliquots of the overlying medium were taken from day 0 (residual inoculum) to 7 post-infection. Each aliquot was 0.5 ml from a total of 2 ml overlay, and was replaced by fresh medium. The titers shown are the means of 3 replicate cultures ± S.E.

ture sensitivity were produced by combining *ts* and non-*ts* mutations.

3.4. Attenuation phenotype of rHPIV1 mutants in hamsters

We next examined the in vivo replicative capacity of selected rHPIV1 vaccine candidates in hamsters (Table 4). Groups of hamsters were immunized i.n. with each of the indicated viruses. HPIV1$_{LLC1}$ grew efficiently in both the URT and the lower respiratory tract (LRT) of hamsters. The rHN^{T553A} mutant grew slightly more efficiently in the LRT compared to HPIV1$_{LLC1}$ (a difference of 0.9 $\log_{10}$ $TCID_{50}$/g) indicating that HN^{T553A} is not an attenuating mutation in hamsters. The rC^{R84G*} was attenuated in the URT and LRT of hamsters compared to HPIV1$_{LLC1}$ ($P<0.001$). However, the rC^{R84G} (rHPIV1$_{LLC4}$) mutant exhibited a similar level of replication to HPIV1$_{LLC1}$, indicating that this combination of mutations specifies a host range *att* phenotype, i.e., *att* in AGMs (Table 1) but not in hamsters (Table 4). When the P/C gene mutations were combined with L gene mutations, either individually (rC^{R84G}L^{L992C}) or in combination (rC^{R84G}L$^{Y942A/L992C}$, rC$^{R84G/F170S}$L$^{Y942A/L992C}$), the vaccine candidates were significantly more attenuated for growth in both the URT and LRT (Table 4). Thus, rHPIV1s bearing combinations of mutations in C and L are highly attenuated in hamsters.

3.5. The effect of mutations in the C and L genes on rHPIV1 replication in AGMs

The attenuation phenotype specified by mutations in the C and L genes was evaluated in AGMs. A rHPIV1 vaccine candidate was considered attenuated if it exhibited a significant ($P<0.05$) reduction in replication in either the mean peak virus titer or the mean sum of the daily virus titers in either the nasopharyngeal NP swab or TL samples compared to the HPIV1$_{LLC1}$ group. As described above, the combination of C^{R84G} and HN^{T553A} mutations specifies an *att* phenotype in AGMs. The rC^{F170S} mutant was attenuated in the URT and LRT, but its attenuation was not additive with that of C^{R84G} and HN^{T553A}, i.e., rC$^{R84G/F170S}$ was not significantly more attenuated than rC^{R84G} (compare groups 4 and 6, Table 5).

Next, individual L gene mutations were inserted onto the rC^{R84G} background, which, as described above, was attenuating only in the URT. Therefore, any attenuation observed in the LRT for a given combination presumably would be due to the added L mutation(s), whereas any attenuating effect of the added L mutation(s) in the URT would be evident only if it was additive to that conferred by the rC^{R84G} backbone. Addition of either of the two non-*ts* L^{L1558I} and L^{L992F} mutations or the *ts* L^{Y942H} mutation to the rC^{R84G} backbone did not confer attenuation in the LRT (Table 5, compare groups 7, 8, and 11 with group 4). In contrast, the individual addition of the L^{L992C}, L^{F456L}, or L^{Y942A} mutation to the rC^{R84G} backbone conferred attenuation for the LRT (Table 5, compare groups 9, 10, and 12 with group 4). Thus, each of the two mutations contained in rC^{R84G}L^{L992C}, rC^{R84G}L^{F456L}, and rC^{R84G}L^{Y942A} contribute to the *att* phenotype of the virus. Next, multiple C and L mutations were combined. The rC^{R84G}L$^{Y942A/L992C}$ and rC$^{R84G/F170S}$L$^{Y942A/L992C}$ recombinants (groups 13 and 14, Table 5) were the most restricted in replication of all of the mutants, with the latter not being recovered from the AGMs. The sequential addition of *ts* and non-*ts* *att* mutations generated a set of rHPIV1 vaccine candidates that exhibit a spectrum of attenuation in AGMs, as illustrated in Fig. 3A.

3.6. In vitro and in vivo stability of the rC$^{R84G/F170S}$L$^{Y942A/L992C}$ vaccine candidate

rHPIV1s bearing multiple mutations that independently contribute to the *att* phenotype would be expected to pos-

Table 4
Replication of rHPIV1 mutants in the upper and lower respiratory tract of hamsters

Virus[a]		No. of animals	Mean virus titer ($\log_{10}$ $TCID_{50}$/g)[b]	
			Nasal turbinates[c]	Lungs[c]
1	HPIV1$_{LLC1}$	10	5.0±0.1	4.5±0.3
2	rHN^{T553A}	6	4.8±0.2	5.4±0.2
3	rC^{R84G*}	6	3.7±0.3	2.9±0.2
4	rC^{R84G}	30	4.7±0.1	4.3±0.2
5	rC^{R84G} L^{L992C}	6	1.7±0.2[d]	1.7±0.2
6	rC^{R84G} L$^{Y942A/L992C}$	5	≤1.5±0.0	≤1.5±0.0
7	rC$^{R84G/F170S}$ L$^{Y942A/L992C}$	12	≤1.6±0.1	≤1.5±0.0

[a] Hamsters in groups of five or six were inoculated i.n. with 10^6 $TCID_{50}$ of the indicated virus. Data are compiled from five independent experiments.
[b] Virus present in the tissues was quantified by serial dilution on LLC-MK2 monolayer cultures at 32 °C and is expressed as $\log_{10}$ $TCID_{50}$/g ± S.E. The limit of detection was 1.5 $\log_{10}$ $TCID_{50}$/g.
[c] Nasal turbinates and lungs from each group were harvested on day 4 post-infection.
[d] Underlined values indicate a significant reduction in virus replication compared to HPIV1$_{LLC1}$, $P<0.01$ (Student–Newman–Keuls multiple comparison test).
* Indicates that this rHPIV1 does not contain the HN^{T553A} mutation, all other recombinant viruses containing the C^{R84G} mutation also contain the HN^{T553A} mutation (see the text).

Table 5
The effect of C and L gene mutations on HPIV1 replication in the lower respiratory tract of African green monkeys[a]

Virus[a]		Shut-off temperature[b] (°C)	No. of animals	Mean peak virus titer (log_{10} $TCID_{50}$/ml)		Mean sum of the daily virus titers (log_{10} $TCID_{50}$/ml)		att[c]	
				NP swab	TL	NP swab	TL	URT	LRT
1	$HPIV1_{LLCI}$[d]	–	8	4.0 ± 0.2	4.3 ± 0.4	25.3 ± 1.7	14.3 ± 1.9		
2	rHN^{T553A}[d]	–	4	4.3 ± 0.2	4.6 ± 0.2	25.4 ± 0.7	13.7 ± 1.8	No	No
3	rC^{R84G*},[d]	–	4	3.6 ± 0.4	4.1 ± 0.5	21.5 ± 1.7	11.7 ± 2.5	No	No
4	rC^{R84G}[d]	–	12	2.1 ± 0.2[e]	4.8 ± 0.3	11.0 ± 0.9[e]	14.3 ± 1.1	Yes	No
5	rC^{F170S}	–	4	3.2 ± 0.9	2.7 ± 0.9[f]	15.9 ± 3.6[e]	6.2 ± 1.3[e,f]	Yes	Yes
6	$rC^{R84G/F170S}$	–	6	1.9 ± 0.3[e]	3.2 ± 0.6	9.4 ± 0.7[e]	9.9 ± 2.5	Yes	No
7	rC^{R84G} L^{L1558I}	–	4	3.4 ± 0.2	4.6 ± 0.3	17.2 ± 1.7[e]	15.0 ± 1.4	Yes	No
8	rC^{R84G} L^{L992F}	–	4	2.7 ± 0.5[e]	5.0 ± 0.5[g]	15.1 ± 2.9[e]	16.9 ± 1.6[g]	Yes	No
9	rC^{R84G} L^{L992C}	38	4	1.4 ± 0.1[e]	0.6 ± 0.1[e,f,g]	7.5 ± 0.7[e]	2.8 ± 0.3[e,f,g]	Yes	Yes
10	rC^{R84G} L^{F456L}	37	4	1.1 ± 0.4[e]	2.6 ± 0.5[f]	7.0 ± 1.0[e]	7.5 ± 1.2	Yes	No
11	rC^{R84G} L^{Y942H}	37	4	1.7 ± 0.2[e]	3.5 ± 0.1	10.8 ± 1.4[e]	9.0 ± 1.0	Yes	No
12	rC^{R84G} L^{Y942A}	37	4	2.0 ± 0.2[e]	2.2 ± 0.6[e,f]	12.0 ± 0.9[e]	4.3 ± 0.6[e,f]	Yes	Yes
13	rC^{R84G} $L^{Y942A/L992C}$	36	4	1.1 ± 0.2[e]	0.8 ± 0.3[e]	6.5 ± 0.4[e]	2.8 ± 0.3[e]	Yes	Yes
14	$rC^{R84G/F170S}$ $L^{Y942A/L992C}$	35	4	≤0.5 ± 0.0[e]	≤0.5 ± 0.0[e]	≤5.5 ± 0.0[e]	≤2.5 ± 0.0[e]	Yes	Yes

[a] Monkeys were inoculated and virus shedding was monitored as described in footnotes a–g of Table 1.
[b] Shut-off temperature is described in footnote c, Table 3.
[c] Virus is designated *att* in the URT or LRT based on a significant reduction in either mean peak titer or mean sum of daily titers compared to the $HPIV1_{LLCI}/rHN^{T553A}$ group (refer to footnote f).
[d] These are the same data as shown in Table 1, repeated here for comparison. Note that $rHPIV1_{LLCI}$ is now designated rC^{R84G} because it contains the *att* combination of C^{R84G} and HN^{T553A} compared to $HPIV1_{LLCI}$.
[e] Statistically significant reduction compared to corresponding $HPIV1_{LLCI}$ titer, $P<0.05$ (Student–Newman–Keuls multiple comparison test).
[f] Statistically significant reduction compared to corresponding rC^{R84G} titer, $P<0.01$ (Student–Newman–Keuls multiple comparison test).
[g] Statistically significant reduction between indicated values, $P<0.001$ (Student–Newman–Keuls multiple comparison test).
[*] Indicates that this rHPIV1 does not contain the HN^{T553A} mutation, all other recombinant viruses containing the C^{R84G} mutation also contain the HN^{T553A} mutation (see the text).

sess enhanced genetic stability. This was investigated for the $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate. This virus was selected for study since it contained two *ts* mutations in the L gene: one, the Y942A mutation, would require three nucleotide substitutions to revert to a codon that does not specify a *ts* phenotype whereas the second, the L992C mutation, could achieve this with a single nucleotide substitution [40]. Note that while Table 2 indicates that the Cys codon (TGC) of L992C differs by at least two nucleotides from any possible codon for the wild type assignment of Leu, we previously identified alternative amino acid assignments at position 992 that confer a wild type-like phenotype and differ from the 992C codon by only a single nucleotide, and thus a wild type-like phenotype can be achieved by a single nucleotide substitution (e.g., TAC, Tyr) [40]. Serial passage in vitro of a virus at increasingly restrictive temperatures is an effective method to assess the level of genetic and phenotypic stability for viruses with a *ts* phenotype [40,47–49]. The $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate was subjected to two experimental procedures: (1) passage at the non-selective permissive temperature of 32 °C for 5–7 days for a total of six passages; and (2) passage at the increasingly restrictive temperatures of 35 °C, 36 °C, and 37 °C for 5–7 days with two passages per temperature. This passage series at restrictive temperature should select for viruses with increased ability to replicate at elevated temperature. Aliquots of virus from various passage levels were analyzed for *ts* phenotype and also were subjected to complete consensus sequencing of the P and L genes (Table 6). $rC^{R84G/F170S}L^{Y942A/L992C}$ passaged successfully at 35 °C and 36 °C but was undetectable following the first passage at 37 °C (passage 5). Thus, virus harvested from the second passage at 36 °C (passage 4) was characterized.

Previously, an rHPIV1 bearing the L^{Y942A} mutation was found to be highly stable following similar passage in tissue culture [40], so it was expected that virus containing this highly-stabilized mutation plus the partially-stabilized L^{L992C} mutation would also be highly phenotypically stable. Following serial passage at 32 °C the level of temperature sensitivity of $rC^{R84G/F170S}L^{Y942A/L992C}$ decreased slightly, with a change in shut-off temperature compared to unpassaged virus from 35 °C to 36 °C. However, this slight change in the level of temperature sensitivity did not coincide with a sequence change in the P or L genes, and may be due to experimental variation. Following passage of $rC^{R84G/F170S}L^{Y942A/L992C}$ at increasingly restrictive temperatures, the shut-off temperature shifted from 35 °C for the unpassaged virus to 37 °C for the passaged virus. This correlated with the acquisition of two amino acid substitution mutations in the L protein (C992Y and N1427D), and there

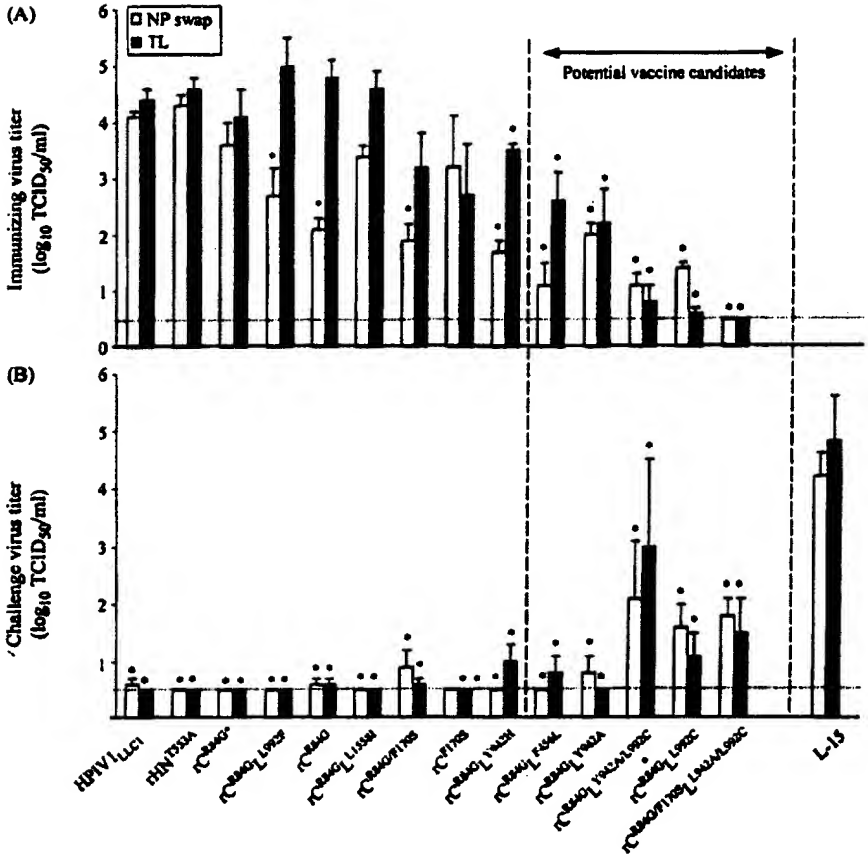

Fig. 3. Comparison of attenuation of replication in AGMs (A) and protective efficacy against $HPIV1_{LLC1}$ challenge (B) for the HPIV1 vaccine candidates indicated along the bottom. Groups of AGMs were inoculated i.n. and i.t. with 10^6 $TCID_{50}$ of $HPIV1_{LLC1}$ or rHPIV1 per site or with medium alone (L-15) as a negative control. (A) Virus titers were determined in the NP swabs and TL samples for each sampling day (see Materials and Methods) and are expressed here as mean peak virus titers (the limit of detection is indicated at 0.5 log_{10} $TCID_{50}$/ml). (B) These AGMs were then challenged i.n. and i.t. with 10^6 $TCID_{50}$ of $HPIV1_{LLC1}$ per site 28 days following the initial immunization, and the titer of the challenge virus shed was determined in the NP swab and TL samples and is expressed here as mean peak challenge virus titer. Significant reductions in $HPIV1_{LLC1}$ titer in rHPIV1 groups vs. the L-15 control are designated by (*) ($P<0.01$). Each rHPIV1 vaccine candidate induced resistance to the replication of $HPIV1_{LLC1}$ challenge virus. However, note that the level of protection against the challenge virus reflected the level of attenuation of the rHPIV1 vaccine candidates, i.e., the greater the level of attenuation, the lower the protective efficacy. The candidates that have potential for further evaluation as vaccine candidates (bracketed by dotted lines) are those that combined a moderate-to-high degree of attenuation in part A with a moderate-to-high degree of protective efficacy in part B.

were no mutations in P (Table 6). Interestingly, a second independent passage of a $rC^{R84G/F170S}L^{Y942A/L992C}$ at elevated restrictive temperature also resulted in the appearance of the C992Y mutation (data not shown).

As noted above, we previously showed that the Tyr-992 assignment, in contrast to the Cys-992 assignment, did not specify a *ts* phenotype in rHPIV1 [40] and differs from the Cys-992 codon by only a single nucleotide (TGC to TAC). This single nucleotide difference accounts for its emergence and subsequent selection during passage at increasingly restrictive temperatures. Since the L992C mutation made a significant contribution to the *att* phenotype in both hamsters and AGMs, it was necessary to investigate the effect that the Cys-992 to Tyr change would have on the *att* phenotype of the $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate. The $rC^{R84G/F170S}L^{Y942A/L992C}$ passaged mutant bearing the C992Y and N1427D substitutions was found to be attenuated in both the URT and LRT of hamsters and demonstrated the same level of attenuation as the original $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate in this animal model (Table 6). Thus, the Cys-992 to Tyr substitution was not accompanied by a change in the level of attenuation for hamsters indicating that the *att* phenotype of $rC^{R84G/F170S}L^{Y942A/L992C}$ was stable following multiple passages at restrictive temperature. In addition, one isolate recovered from hamsters infected with the attenuated $rC^{R84G/F170S}L^{Y942A/C992Y}$ virus was sequenced and the consensus sequence for the P/C gene and L genes was determined in order to assess the stability of the C^{R84G}, C^{F170S} and L^{Y942A} mutations following replication in vivo. The C^{R84G} and C^{F170S} point mutations in the P/C gene and the L^{Y942A} mutation in the L gene were retained in the isolate.

Table 6
In vitro and in vivo stability of the $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate

Virus passaged at indicated temperature[a]	Shut-off temperature (°C)[b]	Virus titer at 32 °C	Reduction in virus titer at indicated temperature compared to 32 °C[c]						Mutations acquired during passage			Mean virus titer in hamsters ($log_{10}TCID_{50}/g$)[d]	
			35 °C	36 °C	37 °C	38 °C	39 °C	40 °C	P gene[e]	L gene[e]		Nasal Turbinates	Lungs
										Amino acid change	# point mutations		
Unpassaged	35	7.7	3.0	3.8	≥6.5	≥6.5	≥6.5	≥6.5	–	–	–	1.8 ± 0.3	≤1.5 ± 0.0
32 °C	36	6.5	1.3	2.0	4.8	≥5.3	≥5.3	≥5.3	–	–	–	Not done	Not done
35–36 °C	37	4.2	0.8	1.0	2.0	≥3.0	≥3.0	≥3.0	–	C992Y N1427D	1 1	1.8 ± 0.2	≤1.5 ± 0.0

[a] Virus was passaged on LLC-MK2 at various temperatures, either at 32 °C for 5–7 day intervals for a total of six passages or at increasing temperatures of 35 °C, 36 °C and 37 °C for 5–7 day intervals with two passages per temperature for a total of four passages (virus was undetectable during the fifth passage, at 37 °C). LLC-MK2 cells were infected at a multiplicity of infection of 0.01 and each passage involved a 1:10 or 1:100 dilution of the medium overlay.
[b] The lowest temperature at which there is a ≥2 log_{10} reduction vs. 32 °C.
[c] Recovered viruses were sequenced to determine adventitious mutations in the P and L genes of HPIV1 (–, no change). The P gene contained no nucleotide substitutions, and the L gene contained two nucleotide substitutions, each giving rise to one of the indicated amino acid substitutions.
[d] Hamsters were inoculated i.n. with 10^6 $TCID_{50}$ of the indicated virus, six animals per group. Nasal turbinates and lung tissues from each group were harvested on day 4 post-infection. Virus present in the tissues was quantified by serial dilution on LLC-MK2 monolayer cultures at 32 °C and is expressed as log_{10} $TCID_{50}/g \pm S.E.$ The limit of detection was 1.5 log_{10} $TCID_{50}/g$.

3.7. Immunogenicity and efficacy of rHPIV1 vaccine candidates

The level of serum HAI and/or neutralizing antibody titers was determined in order to assess the immunogenicity of the rHPIV1 vaccine candidates in AGMs (Table 7). The vaccine candidates with an intermediate level of attenuation (groups 4–10) developed high titers of HAI antibodies, whereas those with more restricted replication in both the URT and LRT did not have detectable HAI titer and had low or undetectable neutralizing antibody titers (groups 11–14).

The efficacy of the rHPIV1 vaccine candidates in AGMs was investigated by challenging immunized and control animals with $HPIV1_{LLC1}$ 28 days following immunization (Fig. 3). All groups of AGMs demonstrated significantly reduced mean peak challenge virus titers compared to the control group, which had received the L-15 medium inoculum (Fig. 3). It is interesting to note that even the $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate, which had not been detected in respiratory secretions from the URT and LRT of AGMs (Table 5), induced a significant reduction in mean peak challenge virus titer of 2.4 log_{10} $TCID_{50}$/ml and 3.3 log_{10} $TCID_{50}$/ml in the URT and LRT, respectively (Fig. 3). Following challenge, HAI titers increased in all of the vaccine groups tested, indicating that despite antibody production following the initial immunization, AGMs were infected with the challenge $HPIV1_{LLC1}$ virus (Table 7). The viruses that exhibit a satisfactory balance between attenuation and efficacy are identified as potential vaccine candidates in Fig. 3.

4. Discussion

Live attenuated intranasal respiratory virus vaccines have a number of desirable properties, including their ability to induce robust local and systemic immune responses. Several approaches have been utilized to generate live attenuated intranasal virus vaccine candidates for HPIV1. One approach involves the use of a related animal virus, e.g., MPIV1 (Sendai virus), to protect against HPIV1 disease in humans. MPIV1 protected experimental animals from subsequent HPIV1 infection [50,51] and was safe in healthy seropositive adults [52]. However, it replicated to similar levels in AGMs and chimpanzees as HPIV1 and, therefore, it was not attenuated in the primates tested [51]. These findings contrast those with the bovine PIV3 vaccine candidate which was attenuated in both rhesus monkeys and chimpanzees [53,54]. Since the safety profile of the MPIV1 vaccine candidate is not established in humans and since the non-human primate data suggests that it likely would require attenuation for use in human infants [51], we are not pursuing this approach. Another approach, which has been available for a relatively short period of time, is the use of reverse genetics to identify mutations that attenuate HPIV1 in vivo and to introduce them singly or in combination into the HPIV1 genome [31,40]. Importantly, this method allows one to manipulate the viral

Table 7
The immunogenicity of candidate HPIV1 virus vaccines in AGMs and the response to challenge with $HPIV1_{LLC1}$

Virus[a]		No. of animals	Serum neutralizing antibody titer (mean reciprocal $\log_2 \pm$ S.E.)[b]		Serum HAI antibody titer (mean reciprocal $\log_2 \pm$ S.E.)[c]	
			Pre-challenge	Post-challenge	Pre-challenge	Post-challenge
1	$HPIV1_{LLC1}$	8	7.5 ± 0.7	6.6 ± 0.7	6.8 ± 0.3[d]	7.3 ± 1.0[d]
2	rHN^{T553A}	4	8.0 ± 0.4	10.0 ± 0.4	–	–
3	rC^{R84G*}	4	3.8 ± 0.9	4.8 ± 1.3	–	–
4	rC^{R84G}	12	5.9 ± 0.6	8.0 ± 0.4	–	–
5	rC^{F170S}	4	7.1 ± 0.5	9.0 ± 0.7	–	–
6	$rC^{R84G/F170S}$	6	5.3 ± 1.2	7.2 ± 0.7	–	–
7	$rC^{R84G}\,L^{Y942H}$	4	6.8 ± 0.2	8.6 ± 0.8	–	–
8	$rC^{R84G}\,L^{L992F}$	4	8.0 ± 0.4	9.8 ± 0.5	–	–
9	$rC^{R84G}\,L^{L1558I}$	4	8.3 ± 0.5	9.0 ± 0.7	–	–
10	$rC^{R84G}\,L^{F456L}$	4	8.3 ± 0.5	9.5 ± 0.9	–	–
11	$rC^{R84G}\,L^{Y942A}$	4	≤1.0 ± 0.0	1.3 ± 0.3	1.3 ± 1.5	4.5 ± 2.2
12	$rC^{R84G}\,L^{L992C}$	4	≤1.0 ± 0.0	2.5 ± 0.5	2.1 ± 2.7	5.9 ± 0.7
13	$rC^{R84G}\,L^{Y942A/L992C}$	4	≤1.0 ± 0.0	4.5 ± 0.9	0.3 ± 0.5	6.7 ± 0.7
14	$r\,C^{R84G/F170S}\,L^{Y942A/L992C}$	4	≤1.0 ± 0.0	5.3 ± 1.5	1.0 ± 2.0	5.8 ± 3.2
15	None (L15)	6	1.2 ± 0.2	6.6 ± 1.1	0.0 ± 0.0	5.8 ± 0.3

[a] Monkeys were inoculated i.n. and i.t. with 10^6 $TCID_{50}$ of the indicated virus with a 1 ml inoculum at each site and challenged 28 days later with 10^6 $TCID_{50}$ $HPIV1_{LLC1}$ in a 1 ml inoculum at each site.

[b] HAI titers to HPIV1 were determined by HAI assay of sera collected at day 28 (pre-challenge) and day 56 (post-challenge) in separate assays following initial inoculation. Titers are expressed as mean reciprocal $\log_2 \pm$ S.E., the limit of detection is 1.0 ± 0.0.

[c] Neutralizing antibody titers to HPIV1 were determined by complement-enhanced neutralization assay of sera collected at day 28 (pre-challenge) and day 56 (post-challenge) in separate assays following initial inoculation. Titers are expressed as mean reciprocal $\log_2 \pm$ S.E. Neutralization titers were not determined for groups designated (–).

[d] Neutralization antibody titers determined for group 1 were performed only for animals originally inoculated with $HPIV1_{LLC1}$ ($n = 8$).

[*] Indicates that this rHPIV1 does not contain the HN^{T553A} mutation, all other recombinant viruses containing the C^{R84G} mutation also contain the HN^{T553A} mutation (see the text).

cDNA, facilitating the introduction of genetically and phenotypically stable codon substitution or deletion mutations that enables us to increase the safety profile of the vaccine candidate. Attenuating *ts* and non-*ts* mutations can be combined to further enhance the genetic and phenotypic stability of the vaccine candidate.

In the present study, the reverse genetics approach has been used to identify mutations in the rHPIV1 C and L proteins that attenuate wild type HPIV1. The F170S amino acid substitution is one of two non-*ts att* mutations in the C protein found to attenuate HPIV1 for replication in AGMs. The F170S mutation was originally identified in MPIV1 [37,55–57]. Mutations in the C proteins of MPIV1 specify a high level of attenuation in mice, likely, in part, by disrupting the ability of these proteins to inhibit the antiviral activities of interferon [37,55–57]. Specifically, it has been shown that MPIV1 C proteins interact with STAT1 to prevent the tyrosine phosphorylation of the STAT1 protein, and this results in disruption of interferon signaling and the induction of an antiviral state [9,58]. The STAT1-binding domain has been localized to the C-terminal end of the C protein, which contains the F170S amino acid substitution [59]. MPIV1 F170S mutants have been shown to lack the ability to bind STAT1 resulting in an inability to inhibit type I interferon responses [59]. Other C mutants which do not bind STAT1 have also been shown to inhibit type 1 interferon responses [60]. MPIV1 C proteins have pleiotropic effects on viral and cellular proteins with which they interact, and the precise mechanisms by which they counteract the innate immune response remain to be elucidated. Because of the high level of amino acid sequence conservation between the C proteins of HPIV1 and MPIV1, it is reasonable to anticipate that the F170S mutation in HPIV1 similarly disrupts the ability of the C protein(s) to bind to STAT1. The F170S mutation was therefore imported from MPIV1 into HPIV1 and was found to attenuate HPIV1 for the URT and LRT of hamsters [31]. This previously tested rHPIV1 (bearing the F170S mutation) was subsequently found to be a triple mutant containing the introduced F170S mutation as well as the pair of spontaneous C^{R84G} and HN^{T553A} mutations that unknowingly were acquired during passage of the parental virus in vitro and was present in the original $rHPIV1_{LLC4}$ backbone (now designated rC^{R84G}). Thus, the individual contribution of the F170S mutation to restriction of replication in vivo remained unknown. The present study extended the previous study in hamsters by demonstrating that the F170S mutation individually, i.e., in the absence of the C^{R84G} and HN^{T553A} mutations, attenuates HPIV1 for the URT and LRT of AGMs. The level of attenuation specified by the F170S mutation in AGMs was substantial with a 10–80-fold reduction in replication occurring in the URT and LRT, respectively. Since the F170S mutation in the C protein of HPIV1 is not accompa-

nied by a mutation in the overlapping P ORF, it is clear that the F170S mutation affects the C protein to attenuate HPIV1 for AGMs. Thus, this non-*ts* *att* mutation will be a valuable mutation to include in a live attenuated HPIV1 vaccine.

The R84G mutation is the second non-*ts* *att* mutation identified in the HPIV1 C protein. This is a spontaneously occurring mutation that, along with HN^{T553A} (rC^{R84G}), specifies a 100-fold restriction of replication of in the URT of AGMs, but not in the LRT. The mechanism by which the R84G mutation in C and the T553A mutation in HN interact to attenuate HPIV1 for AGMs has not yet been identified. This finding was unexpected since there is no precedent for C and HN protein interaction during HPIV1 infection. It is particularly interesting since the T553A substitution occurs in the C-terminal ectodomain of the HN protein, a class II glycoprotein with an N-terminal anchor and cytoplasmic tail. We would expect that if C and HN protein interaction were to occur it would occur intracellularly or inside a virion since the C protein is not known to be on the surface of infected cells or virions. It might be that the C-terminal mutation in HN affects the conformation of the N-terminal end of this protein. Complex interactions between the ectodomain of virion surface glycoprotein (M2) of influenza A virus and an internal protein (M1) have been described [61]. The point mutation that results in the R84G mutation in the HPIV1 C protein also encodes an amino acid substitution (Glu-87 to Gly) in the P protein. Since rHPIV1 viruses bearing the R84G mutation replicate efficiently, we suggest that the function of the P protein is not adversely affected by the Glu-87 to Gly amino acid substitution. The attenuation phenotype specified by rC^{R84G} is therefore likely due to abrogation of a C and HN protein interaction, which may include inhibition of the host innate immune response. Since $rC^{R84G/F170S}$ and rC^{R84G} replicate to similar levels in the URT of AGMs, the *att* phenotype specified by the C^{F170S} mutation was not additive with that of the C^{R84G} and HN^{T553A} mutations, suggesting that these mutations affect a common function in the C protein. However, combining the two non-*ts* *att* mutations in a virus such as $rC^{R84G/F170S}$ should provide enhanced phenotypic stability to the vaccine candidate since both mutations would have to be lost in order to lose the *att* phenotype.

The L protein F456L, Y942H, Y942A, and L992C mutations, which were studied in the rC^{R84G} backbone, were previously found to specify *ts* and *att* phenotypes in hamsters [31,40]. In the present study, these mutations, plus the L1558I mutation, were evaluated for their level of replication in AGMs. Only the F456L, Y942A, and L992C were found to specify an *att* phenotype in AGMs additional to that specified by the rC^{R84G} backbone. The Y942A and L992C mutations specified the highest level of attenuation. Since rHPIV1 mutants bearing these mutations replicate efficiently in vitro at permissive temperature, the sensitivity of the replication of the *ts* rHPIV1 mutants to the warmer temperatures found in the LRT of AGMs most likely contributes to their attenuation for this site. Interestingly, amino acid positions 456, 942 and 992 are situated in regions of L that are highly conserved among members of the *Paramyxoviridae* family [62,63]. Thus five mutations, one in C, one involving a pair of mutations in C and HN, and three in L, have been identified that make independent contributions to attenuation of rHPIV1 for non-human primates.

HPIV1 vaccine candidates with various combinations of the five *ts* and non-*ts* attenuating mutations were generated to determine if the attenuation specified by the individual mutations was additive and to generate rHPIV1 vaccine candidates that exhibit a wide spectrum of attenuation in vivo, and both of these goals were achieved (Fig. 3). Importantly, rHPIV1 mutants bearing various combinations of mutations replicated relatively efficiently at permissive temperature in vitro, an important consideration for the manufacture of a live attenuated virus vaccine. Combining the Y942A and L992C L protein mutations in the rC^{R84G} backbone increased the level of temperature sensitivity of the virus, and interestingly, the addition of the non-*ts* F170S mutation to this combination further enhanced the level of temperature sensitivity of the virus. The C protein is known to bind to the L polymerase subunit to down-regulate RNA synthesis [64–66]. The inclusion of a non-*ts* mutation in C might enhance temperature sensitivity through an interaction of the C and L proteins. However, this remains to be investigated. Although the $rC^{R84G}L^{Y942A/L992C}$ and $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidates were highly attenuated in both hamsters and AGMs and were only weakly immunogenic in AGMs, immunization with either virus provided a significant level of protection in AGMs against wild type HPIV1 challenge. $rC^{R84G}L^{Y942A/L992C}$ and $rC^{R84G/F170S}L^{Y942A/L992C}$ likely will replicate more efficiently in humans, and therefore be more immunogenic, for two reasons. First, these are human viruses and they would be expected to replicate more efficiently in their natural host than in AGMs in which they cause only an asymptomatic infection. Second, these two vaccine candidates are highly *ts* and might replicate more efficiently in humans, which have a lower core body temperature (37 °C) than AGMs (about 39 °C). Thus, the $rC^{R84G}L^{Y942A/L992C}$ and $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidates that appear slightly over-attenuated in AGMs might actually be satisfactorily attenuated and more immunogenic in humans. The properties of a high level of temperature sensitivity, attenuation and efficacy against wild type HPIV1 challenge make $rC^{R84G}L^{Y942A/L992C}$ and $rC^{R84G/F170S}L^{Y942A/L992C}$ promising vaccine candidates.

Phenotypic and genetic stability are important criteria in developing vaccine candidates since mutations arising during replication in vivo can result in loss of the *ts* and *att* phenotypes. An increase in genetic and phenotypic stability should result from the combination of mutations, and these properties were examined for $rC^{R84G/F170S}L^{Y942A/L992C}$. $rC^{R84G/F170S}L^{Y942A/L992C}$ possesses two *ts* mutations: the codon at position 942 would require three nucleotide substitutions to revert to a codon that specifies a ts^+ phenotype whereas this would require only one nucleotide substitution for the mutation at position 992. This vaccine candidate there-

fore provided an opportunity to examine the stability of the *ts* phenotype specified by two mutations with inherent differences in stability that are present in the same virus. The *ts* phenotype of the $rC^{R84G/F170S}L^{Y942A/L992C}$ vaccine candidate was found to be stable following multiple passages at permissive temperature of 32 °C but to undergo partial loss of temperature sensitivity following replication at restrictive temperatures (35 °C–36 °C). This decrease in temperature sensitivity was accompanied by loss of the *ts* mutation at position 992 that resulted from a single nucleotide substitution. In contrast, the *ts* phenotype specified by the stabilized codon at 942 remained unaltered and the codon was unchanged. Thus, the *ts* phenotype specified by the single nucleotide change was unstable, whereas that specified by the three nucleotide change was stable, confirming the value of a three nucleotide codon substitution mutation in contributing to the overall phenotypic stability of the virus [40]. The *att* phenotype of the $rC^{R84G/F170S}L^{Y942A/C992Y}$ partial revertant in hamsters remained stable following passage at restrictive temperature, as expected, since the passaged virus retains the highly attenuating 942 mutation and the two non-*ts att* mutations in the C protein.

The attenuated $rC^{R84G/F170S}L^{Y942A/L992C}$ and $rC^{R84G}L^{Y942A/L992C}$ vaccine candidates investigated in this study did not induce strong serum antibody responses in AGMs, but were protective against HPIV1 wild type challenge as indicated by about a 100-fold reduction in replication of challenge virus in the URT and LRT. The immunological mechanisms underlying this protective effect are undefined. Infection with a parainfluenza virus induces antibody responses both in serum and nasal secretions, the latter being more predictive of resistance to disease in adults than the former [67]. In addition, cell mediated immune responses contribute to the restriction of the replication of parainfluenza viruses in vivo [68–70]. Since infection of animals with HPIV2 or HPIV3 virus does not induce heterologous protection against HPIV1 [3], the homologous protection observed in the present studies induced by rHPIV1 vaccine candidates strongly suggests that it is the result of an adaptive immune response to HPIV1. We suggest that resistance induced by immunization with the highly attenuated $rC^{R84G/F170S}L^{Y942A/L992C}$ or $rC^{R84G}L^{Y942A/L992C}$ vaccine candidates is a combination of humoral and cellular immune mechanisms acting in concert to restrict replication of the HPIV1 challenge virus.

In summary, this study identifies a set of recombinant HPIV1 viruses that exhibit a spectrum of attenuation phenotypes in a non-human primate model ranging from poorly to highly attenuated (Fig. 3). The most highly attenuated vaccine candidate, $rC^{R84G/F170S}L^{Y942A/L992C}$, demonstrates potential as a live attenuated HPIV1 pediatric vaccine candidate. A combination of *ts* and non-*ts att* mutations which individually specify the attenuation phenotype result in a vaccine candidate which is protective against HPIV1 wild type challenge and *att* and *ts* phenotypes that are stable following in vitro replication. Thus, $rC^{R84G/F170S}L^{Y942A/L992C}$ should be considered for trials in humans as a live attenuated intranasal HPIV1 vaccine candidate.

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Recovery of human metapneumovirus from cDNA: optimization of growth in vitro and expression of additional genes

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Abstract

Human metapneumovirus (HMPV) is a recently recognized causative agent of respiratory tract disease in individuals of all ages and especially young infants. HMPV remains poorly characterized and has been reported to replicate inefficiently in vitro. Complete consensus sequences were recently determined for two isolates representing the two proposed HMPV genetic subgroups (Biacchesi et al., Virology 315 (1) (2003) 1). We have developed a reverse genetic system to produce one of these isolates, CAN97-83, entirely from cDNA. We also recovered a version, rHMPV–GFP, in which the enhanced green fluorescent protein (GFP) was expressed from a transcription cassette inserted as the first gene, leaving the 41-nt leader region and first 16 nt of the N gene undisturbed. The ability to monitor GFP expression in living cells greatly facilitated the initial recovery of this slow-growing virus. In addition, the ability to express a foreign gene from an engineered transcription cassette confirmed the identification of the HMPV transcription signals and identified the F gene-end signal as being highly efficient for transcription termination. The ability to recover virus containing a foreign insert in this position indicated that the viral promoter is contained within the 3′-terminal 57 nt of the genome. Recombinant HMPV replicated in vitro as efficiently as biologically derived HMPV, whereas the kinetics and final yield of rHMPV–GFP were reduced several-fold. Conditions for trypsin treatment were investigated, providing for improved virus yields. Another version of HMPV, rHMPV+G1F23, was recovered that contained a second copy of the G gene and two extra copies of F in promoter-proximal positions in the order G1–F2–F3. Thus, this recombinant genome would encode 11 mRNAs rather than eight and would be 17.3 kb long, 30% longer than that of the natural virus. Nonetheless, the rHMPV+G1F23 virus replicated in vitro with an efficiency that was only modestly reduced compared to rHMPV and was essentially the same as rHMPV–GFP. Northern blot analysis showed that the increased number and promoter-proximal location of the added copies of the F and G genes resulted in a more than 6- and 14-fold increase in the expression of F and G mRNA, respectively, and sequence analysis confirmed the intactness of the added genes in recovered virus. Thus, it should be feasible to construct an HMPV vaccine virus containing extra copies of the G and F putative protective antigen genes to increase antigen expression or to provide representation of additional antigenic lineages or subgroups of HMPV.
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Keywords: Metapneumovirus; Paramyxovirus; Recombinant virus; Respiratory disease; Vaccines; Expression vector

Introduction

Human metapneumovirus (HMPV) was first recognized in 2001 in the Netherlands from infants and children experiencing acute respiratory tract disease (van den Hoogen et al., 2001). HMPV has since been isolated in several continents and is thought to be worldwide in prevalence (Ebihara et al., 2003; Freymouth et al., 2003; Jartti et al., 2002; Maggi et al., 2003; Nissen et al., 2002; Peiris et al., 2003; Peret et al., 2002). HMPV was shown to have a very high seroprevalence in samples taken from children and adults over a 40-year period (Boivin et al., 2002, 2003; Ebihara et al., 2003; Freymouth et al., 2003; Maggi et al., 2003; Peret et al., 2002; van den Hoogen et al., 2001), suggesting that it is a newly recognized rather than newly emerging virus. HMPV resembles human respiratory syncytial virus (HRSV) with regard to disease signs and the ability to infect and cause disease in the young infant in particular as well as in individuals of all ages (Boivin et al., 2002; Falsey et al., 2003; Greensill et al., 2003; Maggi et al.,

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2003; Osterhaus and Fouchier, 2003; Peret et al., 2002; Viazov et al., 2003). Even though the epidemiology of HMPV and its impact on human health need to be further studied and defined, it is thought to be an important agent of pediatric respiratory tract disease for which a vaccine should be developed. Live attenuated vaccines are being developed for HRSV and human parainfluenza viruses types 1, 2, and 3, and a live attenuated HMPV vaccine might be appropriate to include as part of this strategy.

HMPV is an enveloped virus with a genome that is a single negative strand of RNA of approximately 13 kb (Biacchesi et al., 2003; van den Hoogen et al., 2001, 2002). It has been classified presumptively, together with avian metapneumovirus (AMPV), in the Metapneumovirus genus, Pneumovirus subfamily, Paramyxovirus family of the Order Mononegavirales. The Pneumovirus subfamily contains a second genus, Pneumovirus, represented by HRSV. A nearly complete genome sequence was determined for the prototype Netherlands 00-1 strain of HMPV (van den Hoogen et al., 2002), and complete genome sequences were determined for two Canadian strains, CAN97-83 and CAN98-75, which represent the two proposed HMPV genetic subgroups (Biacchesi et al., 2003). These studies confirmed that like AMPV, the 3′ to 5′ HMPV gene order is N-P-M-F-M2-SH-G-L. The mRNA encoded by the M2 gene contains two overlapping open reading frames (ORFs) that have the potential to encode separate proteins, M2-1 and M2-2, as is the case for HRSV (Bermingham and Collins, 1999; Collins et al., 1990, 1996; Jin et al., 2000). By analogy to AMPV and HRSV, the HMPV proteins are (listed according to gene order) N, nucleocapsid RNA binding protein; P, phosphoprotein; M, matrix protein; F, fusion glycoprotein; M2-1, transcription elongation factor; M2-2, RNA synthesis regulatory factor; SH, small hydrophobic surface protein; G, major attachment protein; and L, major polymerase subunit. To date, none of these predicted HMPV proteins have been identified or characterized by direct biochemical means, and their functions remain to be confirmed. HMPV has been described as being difficult to isolate and propagate, reflecting its trypsin dependence, slow replication, and limited range of susceptible cell lines (Boivin et al., 2002; van den Hoogen et al., 2001).

To characterize this newly recognized Pneumovirus, we have developed a reverse genetic system based on the CAN97-83 isolate (HMPV83), one of the isolates for which a complete genome consensus sequence was recently published (Biacchesi et al., 2003). The development of this recovery system was facilitated by the expression of enhanced green fluorescent protein (GFP) from an additional gene inserted into the genome of recombinant HMPV (rHMPV), by which the recovery of this slowly growing virus was monitored. We show that rHMPV replicates in cell culture with an efficiency comparable to that of the biologically derived virus, that reasonably efficient growth can be achieved in vitro including in a cell line appropriate for vaccine manufacture, and that additional genes can be accommodated by HMPV with only a small effect on in vitro replication. This provides the basis for developing a live attenuated, multivalent HMPV vaccine.

Results

Construction of a plasmid encoding the full-length HMPV83 antigenomic RNA

A cDNA clone encoding the complete 13335-nt antigenomic RNA of HMPV isolate HMPV83 was constructed as described in Fig. 1. Three overlapping cloned subgenomic fragments were created: fragment 1 contained the leader and the putative N, P, and M genes, bordered on the upstream end by a T7 RNA polymerase promoter (T7p) and on the downstream end by an *Nhe*I site that was created by four nucleotide substitutions in the putative M–F intergenic region as a marker to distinguish between cDNA-derived and biologically derived HMPV (Fig. 1). Three non-viral G residues were added to the 5′ end of the antigenome to enhance promoter efficiency. Fragment 2 included the putative F, M2, SH, and G genes and was bordered on the upstream side by the added *Nhe*I site and on the downstream side by a naturally occurring *Acc*65I site. Fragment 3 consisted of the putative L gene and trailer sequence, bordered on the upstream side by the *Acc*65I site and on the downstream side by part of the hepatitis delta virus ribozyme sequence ending in an *Rsr*II site that occurs naturally within the ribozyme. The antigenomic cDNA was cloned in vector pBSKSII, which supplied the remainder of the hepatitis delta virus ribozyme followed by a terminator for T7 RNA polymerase (T7t) (Durbin et al., 1997a). The sequence of the complete assembled cDNA was confirmed in its entirety, showing that the encoded antigenome (13335 nt long, exclusive of non-viral nt) differed from the consensus sequence of biologically derived HMPV83 (Biacchesi et al., 2003) only by the four nt involved in the *Nhe*I marker.

Construction of pHMPV–GFP

It was anticipated that the poor growth and relative lack of empirical knowledge for HMPV would complicate the recovery and identification of the cDNA-derived virus. Therefore, the pHMPV antigenomic cDNA was modified by insertion of an additional gene encoding the enhanced green fluorescent protein (GFP). This would provide a means to monitor the recovery of rHMPV in living cells at all stages of transfection and passage. One prerequisite for the expression of a foreign ORF inserted into a mononegavirus genome is that it be flanked by appropriate GS and GE signals to direct transcription by the viral polymerase. Putative consensus sequences of the HMPV GS (GGGACAAnTnnnAATG) and GE (AGTTAATTAAAAA) motifs

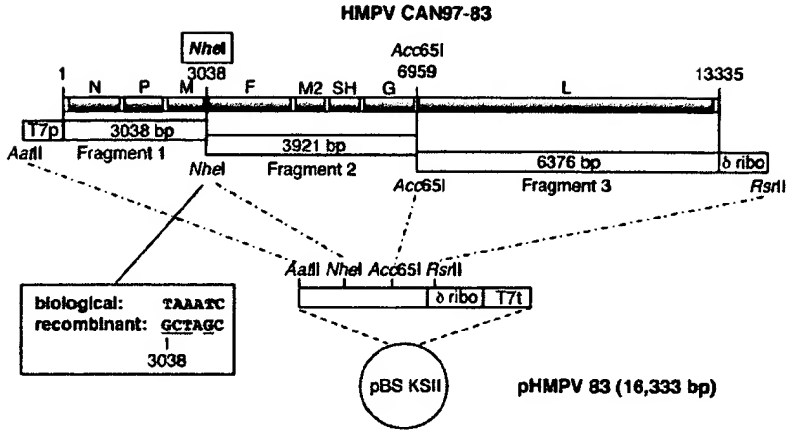

Fig. 1. Construction of plasmid pHMPV83 expressing the complete antigenomic RNA of HMPV83. Three overlapping cDNA fragments (numbered 1–3) covering the complete antigenome were generated by RT-PCR, and were assembled in pBSKSII, which contains the hepatitis delta virus ribozyme (δ ribo) (Perrotta and Been, 1991) followed by a terminator for T7 RNA polymerase (T7t). This vector also contained a polylinker with *AatII*, *NheI*, and *Acc65I* sites, which served to accept the cloned fragments 1, 2, and 3. The complete antigenomic cDNA was designed and confirmed to be identical to the published consensus sequence of biologically derived HMPV83 (Biacchesi et al., 2003) except for 4 nt substitutions (underlined) that were introduced to create an *NheI* marker restriction site in the M-F intergenic region. The leader end of the antigenome cDNA was flanked by the T7 promoter (T7p), and three additional, non-viral G residues were added to the leader end to improve the efficiency of the promoter. The complete pHMPV83 plasmid contains 16333 bp.

were determined previously from sequence alignments of noncoding sequences (Biacchesi et al., 2003; van den Hoogen et al., 2002). The GFP ORF was inserted into the HMPV genome such that its ATG was replaced by that of the N gene. This left undisturbed the 41-nt leader region and first 16 nt of the N gene, including the N GS signal (Fig. 2). The placement of GFP as the first gene should provide for a high level of transcription. The downstream end of the GFP ORF was modified to be followed by the GE signal of the HMPV F gene, and was followed in turn by a 2-nt intergenic region identical to that found between the N and P genes, followed by the complete HMPV N gene. As described for

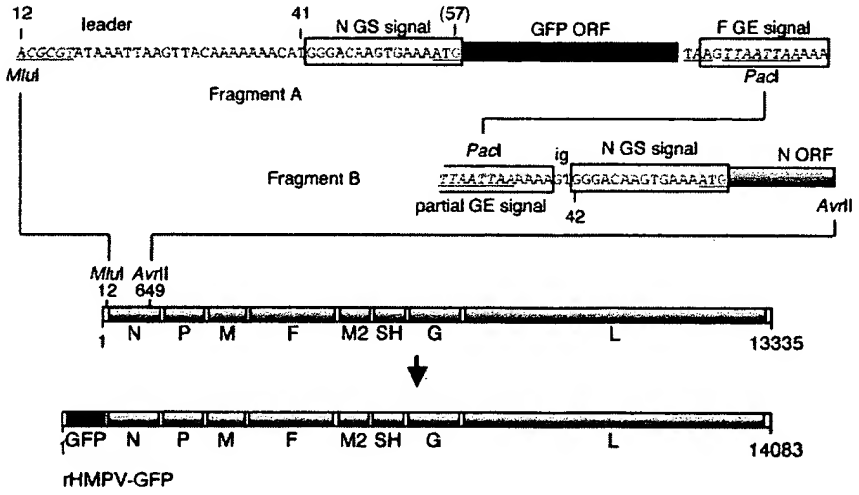

Fig. 2. Insertion of a transcription cassette encoding the green fluorescent protein (GFP) into the promoter-proximal position of HMPV. As described in the Materials and methods, two PCR fragments were constructed: Fragment A contained the GFP ORF (black rectangle) that was flanked on the upstream side by nt 12–57 of the HMPV antigenome, including a naturally occurring *MluI* site, most of the leader region, and the N GS motif. The GFP ORF was designed to initiate with the ATG start codon of the N gene (underlined) and thus is preceded by the first 57 nt of the HMPV antigenome. The GFP ORF was flanked on its downstream side by an HMPV GE motif that was taken from the F gene and contains a naturally occurring *PacI* site. Fragment B contained on the upstream end a partial copy of a GE signal including the *PacI* site, followed by a 2-nt intergenic (ig) region that is identical to that of the N-P junction, followed by a copy of the N GS signal and the upstream end of the N ORF, ending at a naturally occurring *AvrII* site. The two fragments were cloned into the *MluI*–*AvrII* window of the pHMPV antigenome plasmid. In the sequences shown, translational initiation and termination codons are underlined, restriction sites are italicized and underlined, and GS and GE motifs are boxed. The sequence numbering refers to the complete sequence of biologically derived HMPV83 antigenome. The total length of the added GFP transcription cassette (which begins in fragment A after the nucleotide numbered 41 and ends in fragment B before the nucleotide numbered 42) was 746 nt.

pHMPV, the complete sequences of the antigenomic HMPV-GFP cDNA and flanking sequences in pHMPV–GFP also were confirmed by sequence analysis. The length of the encoded rHMPV–GFP antigenome, exclusive of non-viral nt, would be 14083 nt, 5.6% larger than the naturally occurring genome for HMPV83.

Recovery of infectious rHMPV and rHMPV–GFP

The antigenome plasmid pHMPV–GFP was transfected into BSR T7/5 cells, which stably express the T7 RNA polymerase, together with support plasmids encoding the N, P, L, and M2-1 proteins. Two days post-transfection, trypsin was added, the cells were incubated for 3 days and scraped into the medium, and the total suspension was passaged onto LLC-MK2 or Vero cells. When transfected, cells were examined by fluorescent microscopy on successive days post-transfection, green cells were visualized by day 2 that initially consisted of scattered isolated cells and subsequently formed small foci of two or more cells that later on exhibited cytopathic effect consistent with HMPV. When the transfection monolayer was passaged to fresh cells, single green cells were visualized about 24 h post-infection and developed over successive days into multicellular foci. The ability to monitor GFP expression greatly facilitated the initial recovery, which was then also readily achieved for rHMPV lacking the GFP marker.

Infectious rHMPV–GFP also was recovered when the M2-1 support plasmid was omitted from the panel of support plasmids (not shown). This result alone does not necessarily indicate that M2-1 is not required for recovery, because the possibility exists that M2-1 is expressed from the antigenome plasmid, as was found in the case of RSV (Collins et al., 1999). Inclusion of pT7-M2-1 in the transfection had a positive effect on recovery, resulting in two- or threefold more initial recovery events per well. No infectious virus was recovered when the N, P, or L support plasmid was omitted.

The expression of GFP in cells infected with rHMPV–GFP showed that the virus was cDNA-derived and not a contaminating biologically derived HMPV. The rHMPV and rHMPV–GFP viruses were confirmed to be HMPV based on reactivity in both an immunofluorescence assay and a viral plaque immunostaining assay with rabbit antiserum that had been raised against gradient-purified, biologically derived HMPV (data not shown). For rHMPV–GFP, the number of foci of GFP-expressing cells counted before fixation equaled the number of foci that were reactive after immunostaining, showing that the virus population was homogeneous and stably expressing GFP after five passages in cell culture. In addition, RT-PCR performed on virion RNA (vRNA) following three passages in vitro confirmed that both the rHMPV and rHMPV–GFP genomes were efficiently copied and amplified with HMPV-specific primers, that both contained the *Nhe*I restriction marker site whereas biologically derived HMPV did not, and that the rHMPV–GFP virus contained the expected GFP insert (not shown). The expression of the inserted GFP coding sequence also was characterized by Northern blot hybridization. Cells were infected with biologically derived HMPV83, rHMPV, rHMPV–GFP, or were mock infected, and total intracellular RNA was isolated 3 days later and analyzed by Northern blot hybridization with double-stranded DNA probes specific to the GFP or M genes. As shown in Fig. 3, the GFP-specific probe hybridized only with RNA from rHMPV–GFP-infected cells (lane 4), and detected an abundant RNA band of the appropriate size to be the predicted 746-nt (exclusive of polyA) GFP mRNA transcribed from the inserted transcription cassette. This showed that the putative HMPV GS and GE transcription signals

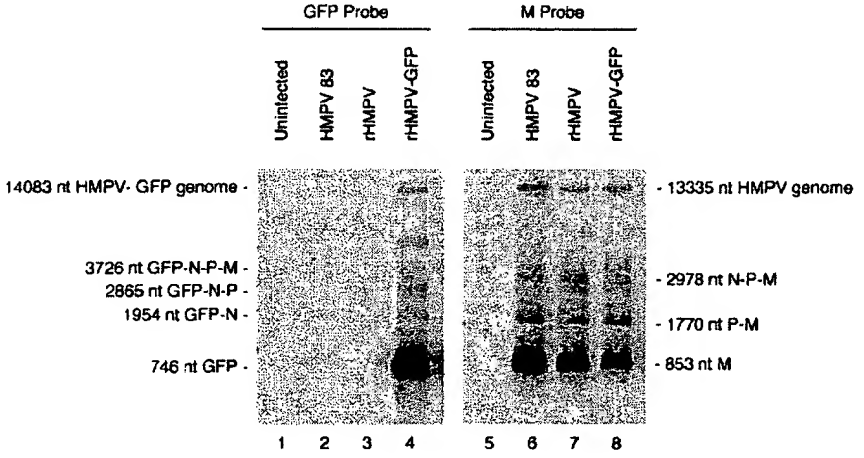

Fig. 3. Northern blot analysis of GFP mRNA expressed by the rHMPV–GFP virus. LLC-MK2 cells were mock-infected (lanes 1 and 5) or infected at an MOI of 3 PFU/cell with HMPV83 (lanes 2 and 6), rHMPV (lanes 3 and 7), or rHMPV–GFP (lanes 4 and 8). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon, and analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the GFP (lanes 1–4) or M (lanes 5–8) gene. The identities and calculated sizes of individual RNA species are indicated.

indeed functioned in the context of the foreign ORF to direct the synthesis of a monocistronic GFP RNA. The GFP probe also hybridized to several larger RNAs that were of low abundance and appeared to represent GFP-N, GFP-N-P, and GFP-N-P-M readthrough mRNAs, as well as to a large, faint band that was of the appropriate size to contain rHMPV–GFP genome and antigenome RNA. Analysis with the M-specific probe identified bands of the appropriate sizes to be the predicted 853-nt monocistronic M mRNA as well as bands representing the P-M and N-P-M readthrough mRNAs. The M probe also detected the genome and antigenome RNA band for each virus, which in rHMPV–GFP would be 748 nt larger than HMPV83 or rHMPV due to the presence of the GFP transcription cassette (Fig. 3, lanes 3, lanes 6, 7, and 8).

The rHMPV virus was compared to its biologically derived HMPV83 parent and to rHMPV–GFP with regard to the efficiency of multicycle replication in vitro in LLC-MK2 cells. As shown in Fig. 4, rHMPV replicated with an efficiency that was similar to that of its biologically derived counterpart. This confirmed that rHMPV was fully competent for multicycle growth, indicating that the previously determined HMPV83 consensus sequence is functional and appears to encode a wild-type virus. HMPV83 and rHMPV reached peak titers 11 days after infection. In comparison, the titer of rHMPV–GFP continued to rise throughout the 13 days of the experiment. Compared to the rHMPV peak titer reached on day 11, the highest rHMPV–GFP titer obtained on day 13 was 7.5-fold reduced. The final titer of rHMPV at day 13 of the experiment was threefold higher than that of rHMPV–GFP. Thus, the insertion of a foreign insert can be accommodated by HMPV without a drastic effect on in vitro replication.

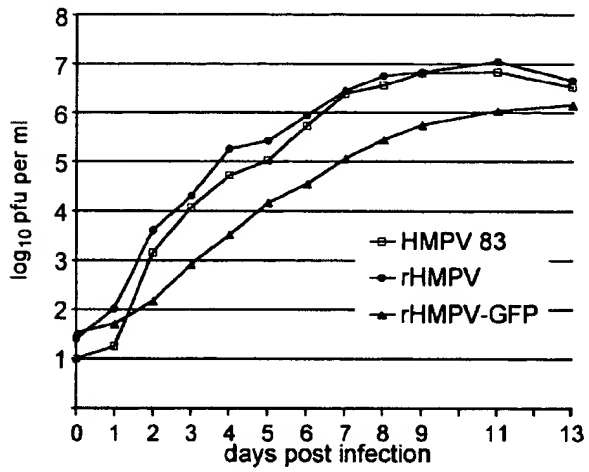

Fig. 4. Comparison of the multi-step growth kinetics of biologically derived HMPV83, rHMPV, and rHMPV–GFP. LLC-MK2 cells were infected at a multiplicity of infection of 0.01 with HMPV83 (□), rHMPV (●), or rHMPV–GFP (▲). Supernatants (0.5 ml out of a total medium volume of 2 ml per well) were taken on indicated days post-infection and replaced by an equivalent volume of fresh medium containing 5 μg/ml of trypsin. The samples were flash frozen and analyzed later by plaque assay and immunostaining. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown.

Optimized trypsin regimen to increase rHMPV–GFP growth

It was previously shown that influenza A virus replication in Vero cells was impaired by rapid inactivation of trypsin in the culture fluids, and that the repeated addition of fresh trypsin to the culture medium restored the multicycle growth pattern and high viral yields (Kaverin and Webster, 1995). We therefore examined the effect of various conditions of trypsin treatment on the growth of rHMPV–GFP. Two different cell lines, LLC-MK2 and Vero, were infected with 0.01 PFU of rHMPV–GFP per cell and incubated at 32 °C with the following trypsin regimen: (i) no trypsin, (ii) 5 μg/ml of trypsin added on day 0 only, 2.5 μg/ml added on day 0 and replenished thereafter at (iii) 24 or (iv) 48 h intervals, or 5 μg/ml added on day 0 and replenished thereafter at (v) 24 h or (vi) 48 h intervals, with the last trypsin addition on day 8 (24 h interval series) or 9 (48 h interval series). Higher levels of trypsin were not tested because of the destructive effect on the cell monolayers. The cells were photographed under fluorescent light at daily intervals up to day 9 to visualize GFP expression; some of these data are shown in Fig. 5A. The medium overlying the cells was completely removed at the time points indicated in Figs. 5B and C up to day 12, and analyzed by plaque titration.

In the absence of added trypsin, the expression of GFP could be visualized by 24 h in a small subset of cells whose numbers appeared to remain essentially constant throughout the course of the experiment (Fig. 5A). It is not known whether the GFP-positive cells observed on, for example, day 9 were the same ones that were initially infected on day 0 or whether they represent a low cycling of cell destruction and reinfection on subsequent days. Plaque titration of the medium overlay harvested at 24 h intervals showed that the release of virus reached a maximum of over 10^3 PFU/ml on day 4 and remained at that approximate level for the remainder of the experiment (Figs. 5B, C). Thus, apparently, a few cells produced virus over this time period that was poorly infectious for multicycle infection in the absence of trypsin but could be scored by plaque assay performed in the presence of trypsin. The results were similar for LLC-MK2 versus Vero cells, although the LLC-MK2 cells yielded somewhat higher titers of virus.

In cells that received a single dose of 5 μg/ml of trypsin on day 0, an increase in the number of GFP-expressing cells was evident with time out to day 12 (day 9 is shown in Fig. 5A). Somewhat surprisingly, the monolayers remained largely intact throughout the 12-day period of incubation despite the large number of infected cells. CPE consisting of scattered enlarged cells with disrupted membrane structures

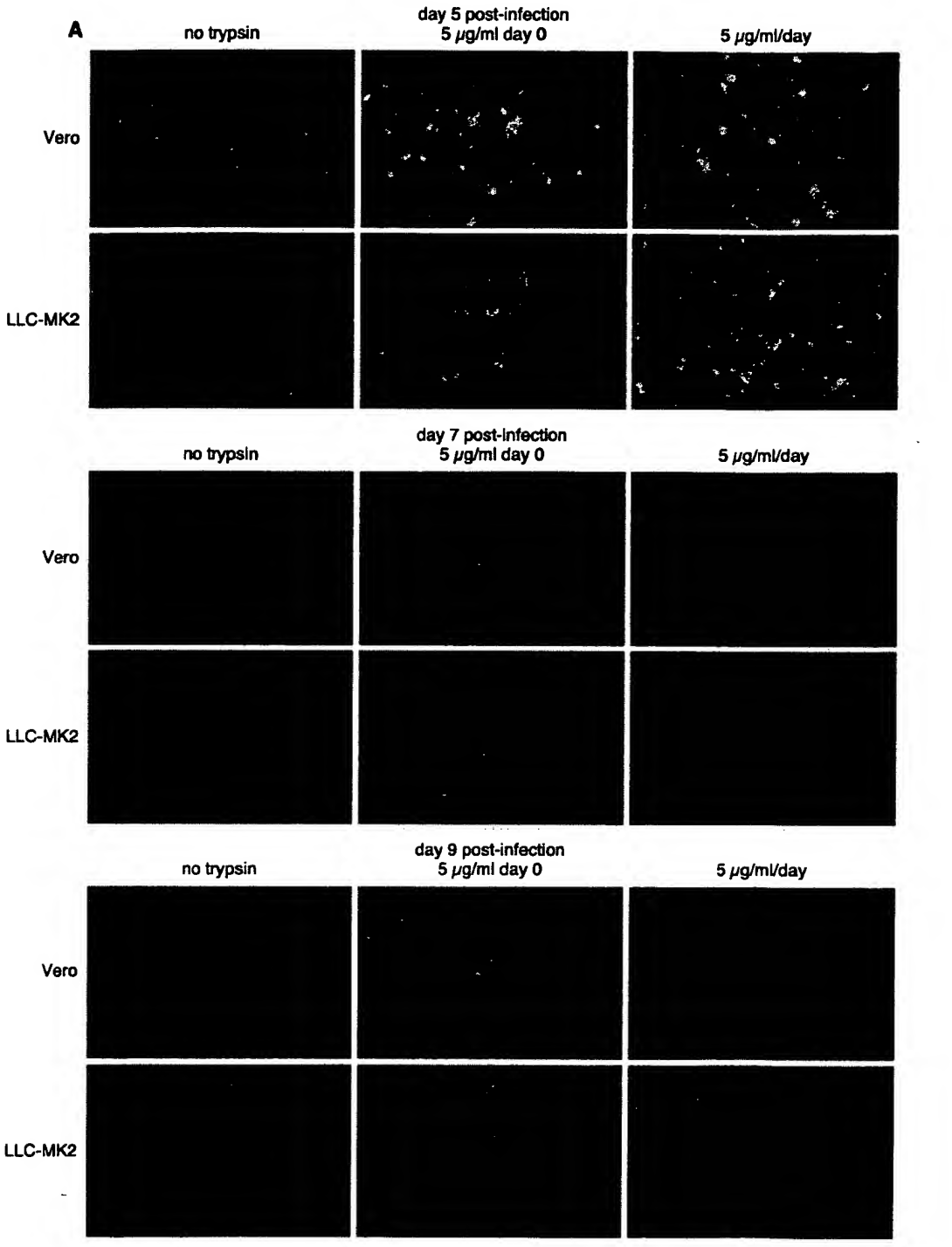

Fig. 5. Effect of different trypsin regimens on the growth of rHMPV–GFP. LLC-MK2 and Vero cells were infected at a multiplicity of infection of 0.01 with rHMPV–GFP and various trypsin regimens were tested. (A) The expression of GFP was monitored daily by fluorescent microscopy. Photographs are shown for 5, 7, and 9 days post-transfection for cultures that received no trypsin, 5 μg/ml of trypsin added on day 0 only, or 5 μg/ml of trypsin added on day 0 and replenished every 24 h. Multi-step growth curves are presented for LLC-MK2 cells (B) and Vero cells (C). Infected cells received no trypsin (■), 5 μg/ml of trypsin added on day 0 only (▲), 2.5 μg/ml of trypsin added on day 0 and replenished, on indicated time points, every 24 h (●) or 48 h (×), or 5 μg/ml of trypsin added on day 0 and replenished, on indicated time points, every 24 h (♦) or 48 h (+) (with the last trypsin addition on day 8 or 9, respectively). The complete medium overlay was harvested at indicated time points. Each time point was represented by two wells, and each virus titration was done in duplicate. The detection limit of this assay is 0.4 $\log_{10}$ PFU per ml of sample. Means are shown.

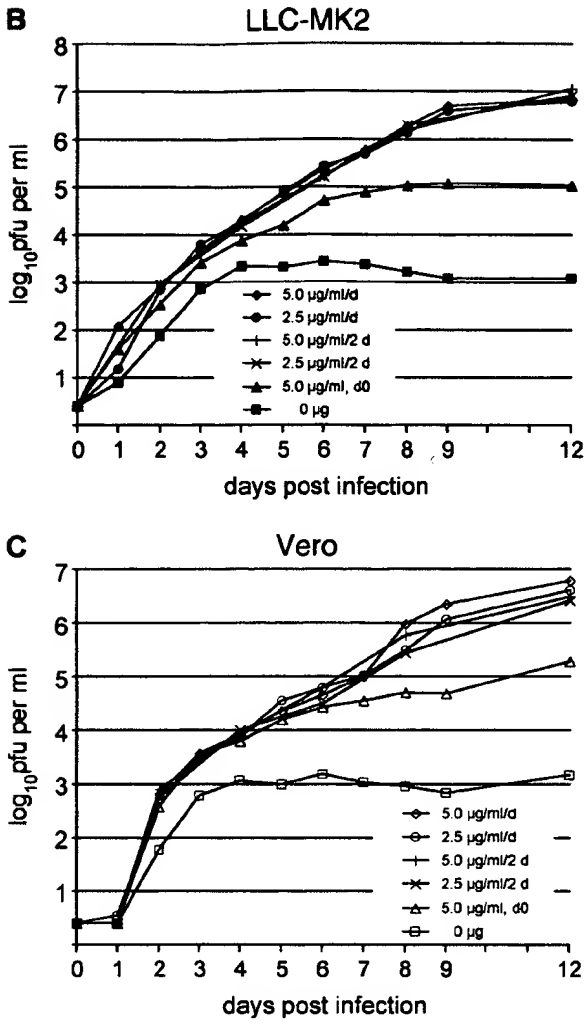

Fig. 5 (*continued*).

was evident by day 9, which later led to detachment of the affected cells from the monolayer (not shown). The formation of multicellular foci was evident by day 3, although large syncytia were not observed. Under these conditions, the titer of released virus reached a maximum of approximately 10^5 PFU/ml on day 7 that was subsequently maintained (Figs. 5B, C). The titers obtained with LLC-MK2 were slightly higher than those with Vero cells. It was somewhat surprising that the release of virus appeared to level off, because the number of GFP-expressing cells appeared to continue to increase; for example, compare days 7 and 9 (Fig. 5A).

Other cells received 5 µg/ml of trypsin on day 0 that was replenished at 24 intervals. This resulted in a faster virus spread and an increasing number of GFP-expressing cells (Fig. 5A) and high titers of released virus (Figs. 5B, C), which in this particular experiment was 6.9×10^6 PFU/ml with LLC-MK2 cells compared with 6.2×10^6 for Vero cells. Similar results were obtained when 2.5 µg of trypsin were used instead of 5 µg. Spread of infection through the Vero cell monolayers was faster as compared with virus spread observed in LLC-MK2 cells. Interestingly, Vero cells consistently produced a greater number of GFP-expressing cells compared to LLC-MK2, but a slightly lower titer of released virus. Small syncytia as shown in Fig. 5A on day 9 were rarely formed in LLC-MK2 cells, but were absent in Vero cells. Both Vero and LLC-MK2 cell monolayers remained largely intact until the experiment was terminated at 12 days. Because of its practical relevance, we also tested the effect of adding 2.5 or 5 µg/ml of trypsin on day 0 with replenishment at 48 h intervals. This resulted in a small (about twofold) drop in final titer in Vero cells, consistent with the greater need for trypsin replenishment noted previously for Vero cells (Kaverin and Webster, 1995), but did not affect the yield in LLC-MK2 cells.

Insertion of multiple extra genes into rHMPV

The insertion of the GFP transcription cassette into rHMPV reduced virus growth only slightly, as described above. It was of interest to investigate the effect of adding multiple, larger extra genes. We therefore replaced the GFP cassette of rHMPV–GFP with a transcription cassette containing a copy of the HMPV G ORF and, in addition, added two copies in tandem of a transcription cassette containing the F ORF in the order G1-F2-F3 (Fig. 6). This resulted in the plasmid pHMPV+G1F23, whose encoded antigenome contains sequences encoding 11 mRNAs rather than the 8 of the natural genome, and would be 17343 nt long, an increase of 30% over the natural genome of 13335 nt. This plasmid was stable in bacteria despite the presence of two identical copies of the G ORF and three identical copies of the F ORF. Recombinant virus was readily recovered from transfections with pHMPV+G1F23, and the virus appeared to replicate with an efficiency similar to that of rHMPV–GFP.

The presence and intactness of the additional genes in the genome of recovered rHMPV+G1F23 were investigated by RT-PCR performed on total RNA from recovered virus following three passages in Vero cells. RT-PCR was performed with a forward primer hybridizing to the HMPV leader region and a reverse primer hybridizing to N sequence, which yielded a single, major band of approximately 4.1 kb, the appropriate size to contain the tandem G1F2F3 supernumerary genes (not shown). The sequence of the supernumerary G1F2F3 genes could not be determined directly by RT-PCR consensus sequencing of viral RNA because primers specific to G or F would prime on each of the two copies of G and three copies of F, precluding analysis of each gene individually. Therefore, we first amplified the supernumerary genes by RT-PCR in three separate, overlapping segments. The first RT-PCR segment used a forward (positive-sense) primer (which also served as the RT primer) from the leader region and a reverse primer from the F gene (840 nt downstream of the F gene start signal): although this latter primer would prime on each copy of F, priming from the promoter-proximal copy (F2, numbered as in rHMPV+G1F23) would be the most efficient in combination with this forward primer, and yielded a ~1.6 kb fragment that contained the leader, the G1 gene, and about 840 bp of the F2 gene, and was purified by gel electrophoresis for sequence analysis. The second segment was amplified by RT-PCR with an RT/forward primer located about 670 nt downstream of the F gene start, and a reverse primer located 554 nt downstream of the F gene start: although the forward primer would prime in all three copies of F and the reverse primer in all three copies of F, priming in F2 and F3 would be the only combination to result in successful amplification of a PCR fragment (~1.5 kb) that contained the downstream 978 bp part of the F2 gene, and a 554 bp upstream part of F3, and was purified by gel electrophoresis for sequence analysis. The third fragment was generated with an RT/forward primer

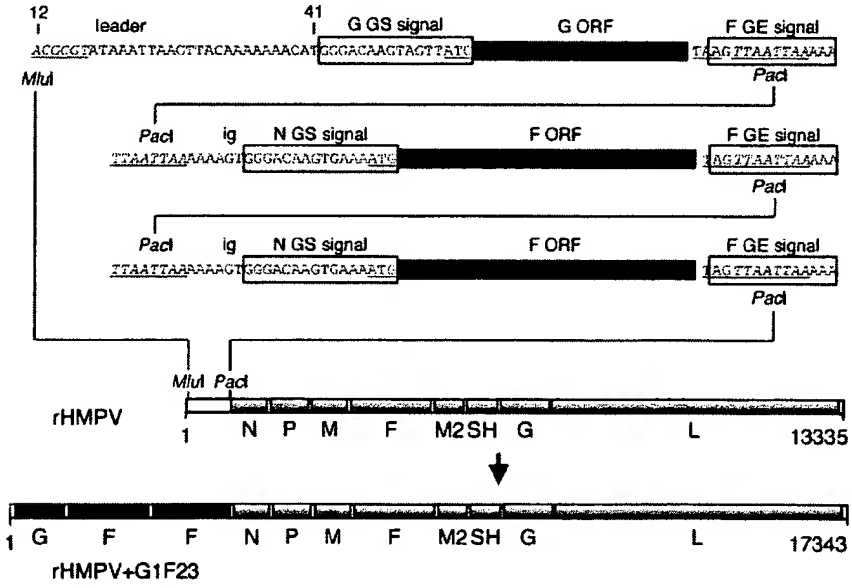

Fig. 6. Insertion of three additional transcription cassettes into rHMPV. The G ORF was modified by PCR to be flanked on the upstream side by nt 12–41 of the leader region including a naturally occurring *MluI* site, and a copy of the G GS signal, and on the downstream side by a copy of the F GE signal including a *PacI* site. The F ORF was modified by PCR to be flanked on the upstream side by a partial GE signal, including a *PacI* site, an intergenic dinucleotide (GT) identical to that normally found between the N and P genes, and a copy of the N GS signal. On the downstream side, the F ORF was flanked by a copy of the F GE signal including a *PacI* site. One copy of the *MluI*–*PacI* G transcription cassette and two copies of the *PacI*–*PacI* F transcription cassette were cloned into the *MluI*–*PacI* window of pHMPV–GFP in the order G-F-F, replacing the GFP transcription cassette. In the sequences shown, translation initiation and stop signals are underlined, restriction sites are italicized and underlined, and GS and GE signals are boxed. The total length of the additional sequence was 4008 nt.

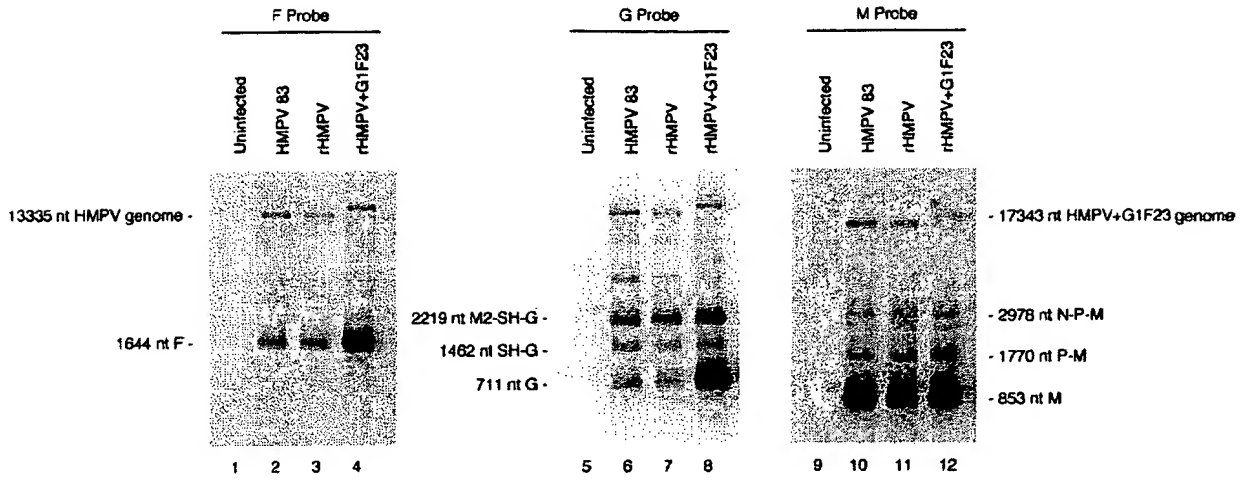

Fig. 7. Northern blot analysis of mRNAs expressed by rHMPV+G1F23. LLC-MK2 cells were mock-infected (lanes 1, 5, and 9) or infected at an MOI of 3 PFU/cell with HMPV83 (lanes 2, 6, and 10), rHMPV (lanes 3, 7 and 11), or rHMPV+G1F23 (lanes 4, 8, and 12). Three days later, total intracellular RNA was isolated, electrophoresed on 1% agarose-formaldehyde gels, transferred to charged nylon, and analyzed by hybridization to double-stranded ^{32}P-labeled DNA probe specific to the F (lanes 1–4), G (lanes 5–8), or M (lanes 9–12) gene. The identities and calculated sizes of individual RNA species are indicated.

from the upstream end of F (14 nt downstream the F gene start signal) and a reverse primer from the upstream end of N: the forward primer would prime in all three copies of F, but priming in F3 would be the most efficient and would yield a ~1.75 kb product that contained most of the F3 gene, and 120 bp of the N gene, and was purified by gel electrophoresis for sequence analysis. This provided an RT-PCR consensus sequence of the G1F2F3 supernumerary genes that was free of mutations, and all GS/GE signals and intergenic sequences were correct, indicating that these added genes were stably recovered in recombinant virus. To determine the effect of the gene additions on viral gene expression, cells were mock-infected or infected with biologically derived HMPV83, rHMPV, or rHMPV+G1F23, and total intracellular RNA was isolated 3 days later and subjected to Northern blot analysis with double-stranded probes to the F, G, or M genes (Fig. 7). The F probe detected a major band of the appropriate size to be the 1644-nt F mRNA, as well as a fainter band of genome and antigenome, which in rHMPV+G1F23 would be 4008 nt larger compared with HMPV83 or rHMPV due to the presence of the three added gene copies (Fig. 7, lane 4). There were only trace amounts of readthrough mRNAs detected with the F probe. Phosphorimager analysis indicated that the amount of genome and antigenome for rHPMV+G1F23 compared to with HMPV83 and rHMPV was 0.9 and 0.5, respectively (corrected for the two extra copies of the F gene present in rHMPV+G1F23). In comparison, the relative amount of F mRNA for rHMPV+G1F23 compared to HMPV83 and rHMPV was six in each case. Normalized to the respective value of genome and antigenome, this corresponded to a 6.6- to 12-fold higher level of F mRNA for rHMPV+G1F23 compared with HMPV83 and rHMPV.

Northern blot analysis with the G probe detected, for each of the three viruses, a band of the appropriate size to be the 711-nt G mRNA as well as bands corresponding to SH-G and M2-SH-G readthrough mRNAs and a band corresponding to antigenome and genome RNAs (Fig. 7, lanes 7, lanes 6, 7, and 8). Phosphorimager analysis indicated that the amount of genome and antigenome for rHMPV+G1F23 compared with HMPV83 and rHMPV was 1.0 and 0.5, respectively (corrected for the extra copy

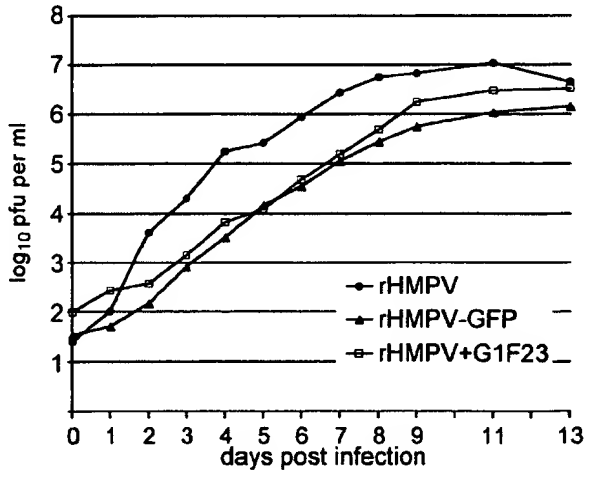

Fig. 8. Comparison of the multi-step growth kinetics of rHMPV, rHMPV–GFP, and rHMPV+G1F23. LLC-MK2 cells were infected at a multiplicity of infection of 0.01 PFU/cell with rHMPV (●), rHMPV–GFP (▲), or rHMPV+G1F23 (□). Medium samples were taken on indicated days post-infection and replaced by an equivalent volume of fresh medium containing 5 μg/ml of trypsin. The samples were flash frozen and analyzed later by plaque assay and immunostaining. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown.

of the G gene present in rHMPV+G1F23), values very similar to that observed with the F probe noted above. In comparison, the relative amount of G mRNA for rHMPV–G1F23 compared with HMPV83 and rHMPV was 14-fold and 15-fold, respectively. This would correspond to a 14- to 30-fold higher level of G mRNA for rHMPV+G1F23 compared with HMPV83 and rHMPV.

The rHMPV+G1F23 virus was compared to rHMPV and rHMPV–GFP with regard to the efficiency of multicycle replication in vitro. LLC-MK2 cells were inoculated with 0.01 PFU/cell and were incubated and sampled in the same manner as for the experiment in Fig. 4. As shown in Fig. 8, rHMPV+G1F23 replicated with an efficiency that was moderately reduced compared with rHMPV but was slightly higher than rHMPV–GFP. Thus, although HMPV is a rather slow-growing virus, it readily accommodates sequence modifications and additions, which opens possibilities for its future use as a vector for mucosal immunization.

Discussion

In this study, we describe the recovery of infectious HMPV from cDNA. HMPV was first recognized in 2001, and the available reports unanimously indicated that it was difficult to isolate and grow in cell culture due to its slow replication, trypsin dependence, small range of susceptible cell lines, and slow development of virus-induced CPE as a marker of virus growth. Therefore, we first constructed a recombinant virus containing an additional transcription cassette encoding GFP, inserted as first promoter-proximal gene. The ability to directly visualize virus spread in living cells compensated technically for the slow development of CPE, and the rHMPV–GFP virus proved to be very helpful both during the establishment of the HMPV reverse genetics system and in optimizing conditions for HMPV replication in vitro. In the future, rHMPV–GFP will also be useful to facilitate diagnostic tests, such as neutralization assays, because this recombinant can be visualized directly. Moreover, rHMPV–GFP will be used to study HMPV tropism and pathogenesis in animal models.

We subsequently recovered rHMPV that differed from the biologically derived virus only by the insertion of an *Nhe*I marker site. This virus replicated in vitro as efficiently as biologically derived HMPV, indicating that it will be appropriate as a starting point to develop a live attenuated HMPV vaccine. The recovery of wild-type-like rHMPV from cloned cDNA provided the first identification of functional sequences for the HMPV genome and its encoded macromolecules. Although the recovery of infectious virus from cloned cDNA has been achieved for several mononegaviruses, HMPV has the distinction of being a recently recognized, poorly characterized agent of human disease, and the development of functional sequences and a reverse genetics system is an important step for molecular epidemiology and vaccine development.

Replenishment of the trypsin in infected cell cultures yielded virus titers that increased over 10–12 days, with a 50- to 100-fold increased final virus yield as compared with titers obtained using a single initial trypsin dose. Trypsin replenishment had a somewhat greater effect on HMPV replication in Vero cells than in LLC-MK2 cells, which is consistent with an observed rapid trypsin inactivation in Vero cell culture fluids (Kaverin and Webster, 1995). From our ongoing experience in propagating HMPV, we have modified this protocol so that 5 μg/ml fresh trypsin is added every second or third day without exchanging the medium. When this regimen is applied during routine production of HMPV stocks, titers of 1.0–4.0 × 10^7 PFU/ml can be routinely achieved in monolayer cultures, which is about 1000-fold improved compared to with titers described in literature (van den Hoogen et al., 2001) and likely could be further improved in optimized cell fermentators. Importantly, these improved titers can be achieved in Vero cells, which are an acceptable substrate for vaccine manufacture. Conditions for efficient replication are important for virological studies in general, and are particularly important for vaccine manufacture to be feasible. These results indicate that it indeed should be feasible to develop and manufacture a live attenuated vaccine for HMPV.

The HMPV CPE that is described in the literature occurs late in infection, on average on day 17 (Boivin et al., 2002) or between day 10 and 14 (van den Hoogen et al., 2001). These observations are consistent with our findings, and the present results indicate that CPE lagged several days behind infection and GFP expression. This was true even when 5 μg/ml of trypsin was added daily, indicating that the slow development of CPE did not reflect a lack of available trypsin. Although these results were obtained using rHMPV–GFP, similar results were obtained with infections involving biologically derived HMPV83 or rHMPV, in which indirect immunofluorescence assays performed on replicate HMPV-infected cells fixed at different time points after infection showed a similar situation in which most of the cells were infected days before the onset of CPE would occur (not shown). This showed that the slow onset of CPE is not due to a reduced replication or restricted spread of rHMPV–GFP.

Detection of GFP mRNA by HMPV showed that the putative N GS and F GE signals that were used to construct the transcription cassette indeed functioned to produce a monocistronic mRNA. The different Northern blot probes used in this study revealed substantial differences in the level of readthrough mRNAs associated with various HMPV genes and the GFP gene. For example, the M gene was associated with a high level of readthrough into downstream genes, whereas the GFP and F genes were associated with little readthrough. The GFP transcription cassette contained the F GE signal, suggesting that this common element is a highly efficient termination signal that is responsible for the lack of observed readthrough mRNAs.

As such, this signal should be optimal for the efficient expression of added genes.

The ability to recover infectious virus containing an insert following nt 57 relative to the 3′ (leader) end of the genome indicates that HMPV, like RSV (Fearns et al., 2000; Fearns et al., 2002), probably has a "short" promoter rather than the dipartite 96-nt promoter that is characteristic of Paramyxovirinae and involves a second promoter element contained within the first gene (Keller et al., 2001; Tapparel et al., 1998). The multi-step growth of rHMPV–GFP in vitro resulted in final titers that were about threefold reduced compared with rHMPV. Because both the rHMPV and rHMPV–GFP full-length plasmids were sequenced in their entirety, and differed only in the presence of the GFP gene, the reduced in vitro growth observed for rHMPV–GFP can be attributed solely to effects from the additional gene. This might indicate that the insert effected a modest reduction in the functioning of a *cis*-acting element such as the promoter. Alternatively, it might be an effect of increased genome length and gene number. The genome of rHMPV–GFP is 748 nt (5.6%) longer compared with rHMPV. In addition to the difference in genome length, the presence of an additional gene as first gene in the genome might contribute to the reduced growth due to attenuation of the transcription of downstream genes.

To further explore the limits of HMPV as a vector, we designed a recombinant virus rHMPV+G1F23 that contains three additional genes in the promoter-proximal position, namely one additional copy of the HMPV G gene and two additional copies of the HMPV F gene. The rHMPV+G1F23 virus has a total genome length that was increased compared with the wild-type virus by approximately 4 kb (30%). The production of infectious virus during multi-step growth in vitro was reduced only modestly compared with rHMPV and was equivalent to or slightly greater than that of rHMPV–GFP. This suggested that although insertion of an additional transcriptional unit into the HMPV genome resulted in a modest decrease in virus replication, this effect was not augmented further by a longer insert or a larger number of transcription cassettes within the range tested. This is offered with the caveat that virus replication might have been enhanced by the expression of the additional copies of G and F, which might have counteracted possible inhibitory effects due to genome length or gene number. Regardless of interpretation, the growth phenotype of rHMPV+G1F23 shows that the HMPV genome is quite versatile, and that the strategy described here can be used to design HMPV vaccine candidates containing additional copies of the putative antigenic determinant genes to achieve an increased gene dose for higher expression. Indeed, Northern blot analysis showed that the expression of F or G mRNA was increased more than 6- or 14-fold, respectively. The approach of adding glycoprotein genes can also be used to generate multivalent rHMPV vaccine candidates expressing antigenic determinants of HMPVs of heterologous antigenic lineages or subgroups in addition to the homologous set of genes present in the recombinant backbone.

It is generally thought that protection against respiratory disease caused by members of the *Pneumovirinae* can be best induced by live attenuated vaccines, particularly given the adverse effects of a previously tested formalin-inactivated HRSV vaccine (Collins and Murphy, 2002). This is where reverse genetic systems have an essential role, as they allow the rational design of safe attenuated vaccine candidates (Collins and Murphy, 2002). HMPV represents an important agent of pediatric respiratory tract disease for which a vaccine should be developed, which will be an important future application of the HMPV reverse genetic system.

Materials and methods

Cells and viruses

LLC-MK2 (ATCC CCL 7.1) and Vero (ATCC CCL-81) cells were maintained in OptiMEM I (Invitrogen GIBCO) supplemented with 5% fetal bovine serum. BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that constitutively express T7 RNA polymerase (Buchholz et al., 1999). They were maintained in Glasgow MEM supplemented with glutamine and amino acids (Invitrogen) and 10% fetal bovine serum. The Canadian HMPV isolate HMPV83 (Peret et al., 2002) and the recombinant HMPVs described below were propagated in Vero, LLC-MK2, or BSR T7/5 cells in the absence of serum and the presence of 5 μg/ml of trypsin. Virus titers were determined by plaque assay on LLC-MK2 cells under methylcellulose overlay containing 5 μg/ml trypsin in the absence of serum, and plaques were visualized 6 days later by immunostaining with a 1:1000 dilution of a polyclonal rabbit serum raised against gradient-purified HMPV83. Growth curves were done with duplicate wells, and each aliquot was titrated in duplicate, and means were calculated.

Viral RNA isolation

Confluent monolayers of LLC-MK2 cells were infected with HMPV83 and incubated at 32 °C in the presence of 5 μg/ml trypsin. Ten days post-infection, clarified supernatants were harvested, and virion RNA (vRNA) was isolated using the QIAamp viral RNA kit (Qiagen) according to the manufacturer's instructions.

Construction of T7 expression plasmids encoding the HMPV antigenome and N, P, M2-1, and L support proteins

A complete cDNA copy of the HMPV83 genome was constructed (Fig. 1) by assembling three overlapping cDNA clones generated by RT-PCR with specific primers designed from the published HMPV83 consensus se-

quence (Genbank accession number, AY297749) (Biacchesi et al., 2003). Primer sequences are available from the authors upon request. First strand cDNA was generated from purified virion RNA using Superscript II reverse transcriptase (Invitrogen). PCR was carried out using Platinum Pfx DNA polymerase (Invitrogen). The nucleotide sequences of cloned RT-PCR fragments were determined using an ABI 3100 sequencer with the Big-Dye terminator ready reaction kit v1.1 (Applied Biosystems). Between two and six clones of each fragment were sequenced completely or in part to identify clones that contained the desired sequence free of error. ORFs encoding the putative nucleoprotein N (1185 nt), phosphoprotein P (885 nt), transcription elongation factor M2-1 (564 nt), and RNA polymerase L (6018 nt) were amplified by RT-PCR from virion RNA using specific primers whose sequences are available from the authors upon request, and were cloned into the T7 expression vector pTM1, wherein translational initiation is mediated by the internal ribosome entry site of encephalomyocarditis virus.

Construction of pHMPV–GFP

The antigenomic pHMPV83 plasmid was modified by the promoter-proximal insertion of a transcription cassette containing the ORF for enhanced GFP (Clonetech, Inc.). This cassette was assembled from two PCR fragments. The first fragment (fragment A in Fig. 2) contained, in 5′ to 3′ order relative to the antigenome, the naturally occurring *Mlu*I site at nt 12 of the leader region, followed by the remainder of the 41-nt leader region, followed by the 16-nt putative N gene-start (GS) sequence (GGGACAAGTGAAA*ATG*, positive sense, HMPV83 nt 42–57, N ORF initiation codon underlined), followed by the GFP ORF that was modified to use the ATG of the N GS signal as its initiation codon, followed by a 14-nt putative gene-end (GE) sequence derived from the F gene (AG*TTAATTAA*AAAA), containing a naturally occurring *Pac*I site (underlined). The second fragment (fragment B in Fig. 2) contained, in 5′ to 3′ order, a partial GE signal containing a *Pac*I site, a 2-nt (GT) intergenic region identical to that of the N–P gene junction, and the N GS signal and partial N gene up to the naturally occurring *Avr*II site at HMPV83 nt 649. Fragments A and B were digested by *Mlu*I/*Pac*I and *Pac*I/*Avr*II, respectively, and cloned simultaneously into an intermediate construct containing cDNA fragment 1 of pHMPV83 (Fig. 1) that had been digested with *Mlu*I/*Avr*II. This modified cDNA fragment 1 was then substituted into the *Aat*II–*Nhe*I window of the complete pHMPV83 antigenomic cDNA clone, leading to the final construct pHMPV–GFP. The length of the encoded rHMPV–GFP antigenome, exclusive of non-viral sequence, would be 14083 nt. The sequences of the regions that had been subjected to PCR, namely the leader, GFP, and upstream end of the N gene, were confirmed by nucleotide sequencing.

Construction of pHMPV+G1F23

cDNAs of the HMPV G or F ORF were amplified by PCR using primers that placed a G GS signal and an F GE signal at the beginning and end, respectively, of the G ORF, or an N GS signal and F GE signal at the beginning and end of the F ORF. In each case, this was followed by a GT dinucleotide intergenic region; in addition, the G transcription cassette was flanked by *Mlu*I and *Pac*I sites, and the F cassette by *Pac*I sites. The *Mlu*I–*Pac*I G fragment was used to replace the *Mlu*I–*Pac*I GFP fragment (Fig. 2) from an intermediate construct containing fragment 1 (comprising the antigenome from leader to *Nhe*I site) of pHMPV–GFP. Next, two copies of the F fragment were cloned into the *Pac*I site of this intermediate plasmid, and restriction digestion was used to identify a clone in which the ORFs of both fragments were in the correct orientation. This G1F23-bearing intermediate fragment was combined with pHMPV fragments 2 and 3 (Fig. 1) to yield pHMPV+G1F23. The length of the encoded rHMPV+G1F23 antigenome, exclusive of non-viral sequence, would be 17343 nt. The sequences of the G and F cassettes were confirmed before assembly by nucleotide sequencing of intermediate clones each bearing an individual cassette.

rHMPV recovery

Confluent BSR T7/5 cells in 6-well dishes were transfected with 5 μg of antigenomic plasmid pHMPV, pHMPV–GFP, or pHMPV+G1F23, 2 μg each of pT7-N and pT7-P, and 1 μg each of pT7-M2-1 and pT7-L support plasmids per well. Transfections were done with Lipofectamine 2000 (Invitrogen) in OptiMEM without trypsin or serum and maintained overnight at 32 °C. The Lipofectamine transfection medium was removed 1 day later and replaced with Glasgow MEM without trypsin or serum. Trypsin was added on day 3 to a final concentration of 5 μg/ml, and cell-medium mixtures were passaged onto fresh LLC-MK2 cells on day 6. The expression of GFP was monitored by fluorescent microscopy.

Northern blot analysis

LLC-MK2 cells were mock-infected or infected with HMPV83 or the indicated recombinant virus, each at an input MOI of 3 PFU/cell, incubated for 3 days, and processed to purify total intracellular RNA using an RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was electrophoresed in a 1% agarose gel in the presence of 0.44 M formaldehyde, transferred to charged nylon membrane (Hybond-N+, Amersham Biosciences), fixed by UV cross-linking, and analyzed by hybridization with denatured double-stranded ^{32}P-labeled DNA probes generated by random priming from GFP, M, G, or F gene cDNA fragments. Radioactivity was detected by analysis with a phosphorimager.

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Recombinant Human Metapneumovirus Lacking the Small Hydrophobic SH and/or Attachment G Glycoprotein: Deletion of G Yields a Promising Vaccine Candidate

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Human metapneumovirus (HMPV) has recently been identified as a significant cause of serious respiratory tract disease in humans. In particular, the emerging information on the contribution of HMPV to pediatric respiratory tract disease suggests that it will be important to develop a vaccine against this virus for use in conjunction with those being developed for human respiratory syncytial virus and the human parainfluenza viruses. A recently described reverse genetic system (S. Biacchesi, M. H. Skiadopoulos, K. C. Tran, B. R. Murphy, P. L. Collins, and U. J. Buchholz, Virology 321:247-259, 2004) was used to generate recombinant HMPVs (rHMPVs) that lack the G gene, the SH gene, or both. The ΔSH, ΔG, and ΔSH/G deletion mutants were readily recovered and were found to replicate efficiently during multicycle growth in cell culture. Thus, the SH and G proteins are not essential for growth in cell culture. Apart from the absence of the deleted protein(s), the virions produced by the gene deletion mutants were similar by protein yield and gel electrophoresis protein profile to wild-type HMPV. When administered intranasally to hamsters, the ΔG and ΔSH/G mutants replicated in both the upper and lower respiratory tracts, showing that HMPV containing F as the sole viral surface protein is competent for replication in vivo. However, both viruses were at least 40-fold and 600-fold restricted in replication in the lower and upper respiratory tract, respectively, compared to wild-type rHMPV. They also induced high titers of HMPV-neutralizing serum antibodies and conferred complete protection against replication of wild-type HMPV challenge virus in the lungs. Surprisingly, G is dispensable for protection, and the ΔG and ΔSH/G viruses represent promising vaccine candidates. In contrast, ΔSH replicated somewhat more efficiently in hamster lungs compared to wild-type rHMPV (20-fold increase on day 5 postinfection). This indicates that SH is completely dispensable in vivo and that its deletion does not confer an attenuating effect, at least in this rodent model.

The newly discovered human metapneumovirus (HMPV) appears to be a major etiologic agent for lower respiratory tract disease in children (10, 32). HMPV was first identified in The Netherlands in 2001 (30) and soon after was isolated in patients with respiratory tract disease throughout the world (16). HMPV is a member of the *Metapneumovirus* genus of the subfamily *Pneumovirinae*, family *Paramyxoviridae*, order *Mononegavirales*. Human respiratory syncytial virus (HRSV), a member of *Pneumovirinae* that has been studied in the greatest detail, belongs to a second genus, *Pneumovirus*.

The HMPV genome, which ranges in length from 13,280 to 13,335 nucleotides (nt) for the available complete sequences (1), contains eight genes in the order 3′-N-P-M-F-M2-SH-G-L-5′ and encodes nine proteins (1, 29). By analogy to HRSV, the HMPV proteins are the following: N, nucleoprotein; P, phosphoprotein; M, matrix protein; F, fusion protein; M2-1, transcription elongation factor; M2-2, RNA synthesis regulatory factor; SH, small hydrophobic protein of unknown function; G, attachment glycoprotein; and L, viral polymerase. Compared to HRSV, HMPV lacks the nonstructural NS1 and NS2 genes and has the F, M2, SH, and G genes in the order F-M2-SH-G, compared to SH-G-F-M2 for HRSV.

Two genetic subgroups have been described for HMPV which are 80% identical on the nucleotide level and 90% identical on the aggregate amino acid level (1). These values are similar to the 81% nucleotide identity and 88% aggregate amino acid sequence identity observed for the two subgroups of HRSV (A2 and B1 strains; GenBank accession nos. M74568 and AF013254, respectively). It was initially reported that these two HMPV genetic subgroups represented two serotypes (31). However, other studies indicated that the known isolates of HMPV formed a single serotype and that the two genetic subgroups exhibited less antigenic diversity than was the case for the two subgroups of HRSV (15, 22). Members of the *Pneumovirinae* subfamily (hereafter called pneumoviruses) encode three surface glycoproteins, F, G, and SH (5). The fusion glycoprotein F, which is highly conserved (95% identity between the two HMPV subgroups, compared to 89% between the HRSV subgroups), mediates penetration and syncytium formation. The human and bovine RSV F proteins are synthesized as an F_0 precursor that is cleaved intracellularly by a furin-like protease at two closely spaced sites to yield the F_1 and F_2 subunits (5) plus an intervening peptide (9, 34). The

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HMPV F_0 protein contains a single candidate cleavage site whose structure, R-Q-X-R, does not conform to the furin motif, and HMPV usually has been reported to require exogenous trypsin for growth in vitro. The HRSV F protein is one of the two major neutralization and protective antigens of HRSV, and HMPV F also appears to be an important HMPV neutralization and protective antigen (22, 24).

The pneumovirus G protein is a type II membrane protein, such that its membrane anchor is proximal to the N terminus and its C terminus is oriented externally. The G protein of HRSV has been studied in the greatest detail among the pneumoviruses. Most of its ectodomain has a high content of serine, threonine, and proline residues and numerous O- and N-linked sugars, features that are reminiscent of mucins and might confer an extended, nonglobular structure (5). The HRSV G protein is 289 to 299 amino acids (aa) in length and is highly divergent among HRSV strains (55% identity between the two HRSV antigenic subgroups) (11, 23), a characteristic that is even more pronounced for HMPV G (31 to 35% identity between the two proposed genetic subgroups), with an amino acid length of 217 to 236 (1, 18). The high carbohydrate content of HRSV G results in an apparent molecular mass as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 84 to 90 kDa compared to 32.6 kDa for the unmodified form calculated from the gene sequence. Remarkably, HRSV G is not essential for virus assembly (27) or growth in vitro. Indeed, a biologically derived, cold-passaged, attenuated HRSV subgroup B mutant (B1 cp-52) was shown to replicate to high titers in cell culture despite the absence of functional SH and G proteins (13). Moreover, recombinant HRSV (rHRSV) from which the G gene alone was deleted was infectious but had an in vitro host range restriction and was highly attenuated in the respiratory tract of mice. Virus could not be recovered from the nasal turbinates, only trace amounts were detected in the lungs, and it was uncertain whether replication had occurred (26, 28). Comparable results were observed for a recombinant bovine RSV (BRSV) lacking its G gene which was fully competent with regard to in vitro growth and strongly attenuated in the respiratory tract of calves (12, 21).

The pneumovirus SH protein also is a type II transmembrane glycoprotein (6). The HMPV SH protein is the largest among the pneumoviruses (179 aa for the Canadian HMPV isolate CAN97-83 [HMPV83], versus 175 aa for avian metapneumovirus(MPV) C, 174 aa for AMPV A, 81 aa for BRSV, and 64 aa for HRSV A2) (1, 29). The HMPV SH protein is poorly conserved (59% identity between the two proposed genetic subgroups) and is more divergent than its HRSV counterpart (72% identity between subgroups). In HRSV-infected cells, SH accumulates in several different forms: SH0 (mass, 7.5 kDa) is the full-length nonglycosylated species, SHg (13 to 15 kDa) contains one high-mannose N-linked carbohydrate chain, SHp (21 to 30 kDa) is generated by the addition of polylactosaminoglycan to the N-linked carbohydrate chain, and SHt (4.8 kDa) is a nonglycosylated species that is derived from an internal initiation (17). SH0 and SHp are the predominant forms in the virion. On the basis of chemical cross-linking (6) and global searching of molecular dynamic simulations (14), SH was suggested to form channel-like homopentamers whose minimal transmembrane pore is 1.46 Å. These observations are reinforced by the observation that expression of the SH protein in bacteria increases membrane permeability to small-molecular-weight compounds (20). However, the function of SH in the viral infectious cycle still remains unknown. Recombinant bovine and human RSV from which the SH gene has been deleted (ΔSH) are fully viable in cell culture (4, 12). In vivo, the HRSVΔSH virus was attenuated in the upper, but not in the lower, respiratory tract of mice and was slightly attenuated at both sites in chimpanzees (4, 33).

The ability to produce infectious rHMPV from full-length cDNA (2) provides a method to investigate the functions of individual HMPV proteins and to develop live-attenuated vaccines. As a first step for functional studies and the development of attenuated HMPV derivatives, we engineered a full-length cDNA copy of the HMPV antigenome to delete the SH, G, or both SH and G genes together in their entirety. Recombinant virus was recovered and analyzed in vitro and by infection in hamsters.

MATERIALS AND METHODS

Cells and viruses. LLC-MK2 cells (ATCC CCL 7.1) were maintained in OptiMEM I (Invitrogen GIBCO) supplemented with 5% fetal bovine serum and 2 mM L-glutamine. BSR T7/5 cells are baby hamster kidney 21 (BHK-21) cells that constitutively express T7 RNA polymerase (3). They were maintained in Glasgow minimal essential medium (MEM) supplemented with 2 mM L-glutamine and amino acids (1× final MEM amino acids solution; Invitrogen), 10% fetal bovine serum, and 1 mg of Geneticin/ml. The Canadian isolate HMPV83 (19) and the rHMPVs (all derived from HMPV83) were propagated in LLC-MK2 cells at 32°C in the absence of serum and the presence of 5 μg of trypsin/ml. Virus titers were determined by plaque assay on LLC-MK2 cells under methylcellulose overlay containing 5 μg of trypsin/ml as described previously (2). Plaques were visualized 6 days later by immunostaining with an anti-HMPV83 polyclonal rabbit antiserum (2), and the final titer was expressed in PFU per milliliter. Alternatively, virus titers were determined by serial 10-fold dilutions of virus applied to LLC-MK2 monolayer cultures on 96-well plates. Cultures were incubated for 6 days at 32°C in OptiMEM medium with 5 μg of trypsin/ml, and infected wells were detected by immunostaining as described above. The final titer was expressed in 50% tissue culture infective doses ($TCID_{50}$) per milliliter.

Construction of SH and/or G gene deletion mutant cDNA. The plasmid pHMPV, which contains the complete consensus antigenomic sequence of HMPV83 (GenBank accession number AY297749 [1]) except for four nucleotide substitutions involved in the NheI site (nt 3038) as described previously (2), was modified to contain an additional BsiWI and BsrGI restriction site in the M2-SH and SH-G intergenic regions, respectively (Fig. 1). To introduce the restriction sites, the NheI/Acc65I fragment of pHMPV, containing the F, M2, SH, and G genes, was subcloned, and a two-step site-directed mutagenesis was done using the QuikChange kit (Stratagene). First, the BsiWI restriction site (nt 5459) was created using two complementary primers CTTAAGTTAGTAAAAACA**CGTA CG**AGTGGGATAAGTGACAATG and CATTGTCACTTATCCCACT**CGTA CG**TGTTTTTACTAACTTAAG (mutated nucleotides are in bold, and the BsiWI restriction enzyme site is underlined). Next, the BsrGI restriction site (nt 6099) was created by using the complementary primers GTTTAGTTATTTTA AAATATT**TGTACA**TAGGTAAGTTTCTATGGCAC and GTGCCATAGAA ACTTACCTA**TGTACA**AATATTTTAAAATAACTAAAC (mutated nucleotides are in bold, and the BsrGI restriction enzyme site is underlined). The mutated subclone was then digested with NheI/BsiWI and the resulting fragment was cloned into the NheI/Acc65I window of pHMPV to create pHMPV-ΔSH/G, or it was digested with NheI/BsrGI and the resulting fragment was cloned into the NheI/Acc65I window of pHMPV to create pHMPV-ΔG. To create pHMPV-ΔSH, the mutated subclone was digested with BsiWI/BsrGI, religated, and then digested with NheI/Acc65I, and the resulting fragment was cloned in the NheI/Acc65I window of pHMPV. The strategy for these ligations was based on the knowledge that digestion with BsiWI, BsrGI, and Acc65I creates a common overhang, GTAC. The sequence of the NheI/Acc65I fragment of each modified pHMPV was confirmed by nucleotide sequencing performed with an ABI 3100 automatic sequencer, using the Big-Dye Terminator Ready Reaction kit version 1.1 (Applied Biosystems) and specific primers.

rHMPV recovery. As described previously (2), confluent BSR T7/5 cells in six-well dishes were transfected with 5 μg of antigenomic plasmid pHMPV,

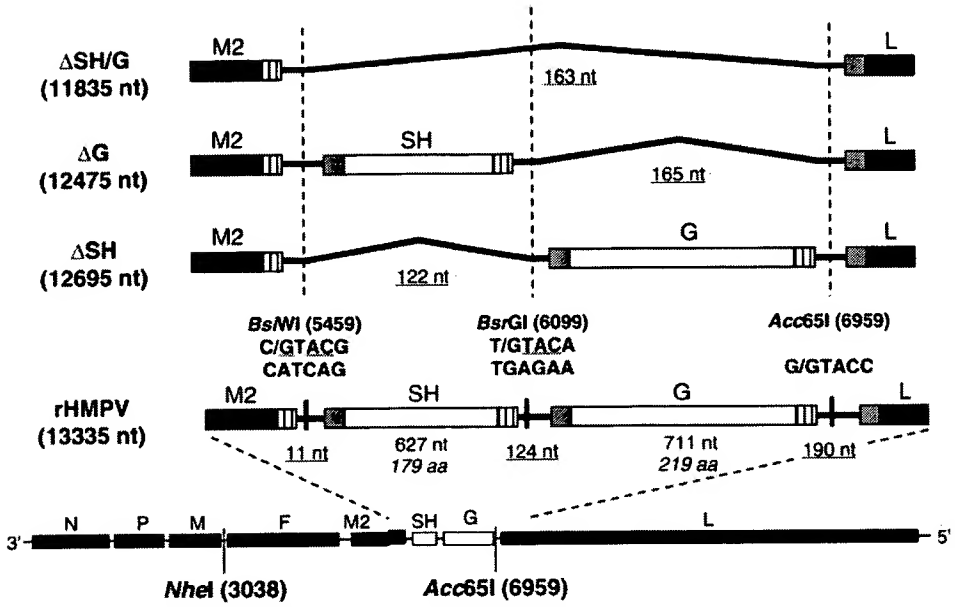

FIG. 1. Construction of the ΔSH, ΔG, and ΔSH/G gene deletion HMPV mutants. The wild-type HMPV genome is shown at the bottom, drawn approximately to scale. A unique NheI site that had been added as a marker (2) is shown, as well as a naturally occurring unique Acc65I site. The enlarged diagram (not to scale) shows the SH-G genome region. The SH and G genes are shown as open rectangles flanked on the upstream and downstream ends by the gene start (shaded boxes) and gene end (hatched boxes) transcription signals, respectively. The nucleotide length of each gene is listed underneath it, and the calculated amino acid length of its encoded protein is given in italics. Intergenic regions are shown as horizontal lines, with nucleotide lengths indicated (underlined). The lengths of new intergenic regions created by the gene deletions also are indicated. Also shown are BsiWI and BsrGI sites that were introduced into the M2-SH and SH-G intergenic regions, respectively: the sequence and nucleotide position of each site (based on the position of its first residue in the antigenomic sequence) are shown, with nucleotide substitutions made to create the sites underlined and the wild-type sequence shown underneath. The genome lengths of the recombinant viruses are indicated to the left.

pHMPV-ΔSH, pHMPV-ΔG, or pHMPV-ΔSH/G, 2 μg each of pT7-N and pT7-P, and 1 μg each of pT7-M2-1 and pT7-L support plasmids per well. Transfections were done with Lipofectamine 2000 (Invitrogen) in OptiMEM without trypsin or serum (day 1) and maintained overnight at 32°C. The Lipofectamine transfection medium was removed (day 2) and replaced with Glasgow MEM without trypsin or serum. Trypsin was added on day 3 to a final concentration of 5 μg/ml, and cell-medium mixtures were passaged onto fresh LLC-MK2 cells on day 4 and incubated at 32°C. The recovered rHMPV, rHMPVΔSH (ΔSH), rHMPVΔG (ΔG), and rHMPVΔSH/G (ΔSH/G) viruses then were amplified by two passages in LLC-MK2 cells, and only low-passage stocks were used in all experiments.

Verification of recombinant viral genome structures. Viral RNA was extracted from each virus stock corresponding to the second passage of rHMPV, ΔSH, ΔG, or ΔSH/G (QIAamp viral RNA kit; QIAGEN). The viral RNA was copied and amplified by reverse transcription-PCR with a positive-sense primer designed to hybridize within the M2 gene (HMPV83 nt 5363 to 5382) and a negative-sense primer designed to hybridize within the L gene (HMPV83 nt 7499 to 7479). PCR products were analyzed on a 1% agarose gel and were also subjected to direct nucleotide sequencing in their entirety.

Northern blot analysis. LLC-MK2 cells were mock infected or infected with rHMPV, ΔSH, ΔG, or ΔSH/G virus, each at an input multiplicity of infection (MOI) of 3 PFU per cell, incubated for 3 days at 32°C in the presence of 5 μg of trypsin/ml, and processed to purify total intracellular RNA using an RNeasy kit (QIAGEN) according to the manufacturer's instructions. RNA was electrophoresed in a 1% agarose gel in the presence of 0.44 M formaldehyde, transferred to a charged nylon membrane (Hybond-N^+; Amersham Biosciences), fixed by UV cross-linking, and analyzed by hybridization with denatured double-stranded ^{32}P-labeled DNA probes generated by random priming from F, SH, or G gene cDNA fragments (Megaprime DNA labeling system; Amersham Biosciences). Radioactivity was detected by analysis with a phosphorimager.

Virus purification by sucrose gradient centrifugation. LLC-MK2 cells were infected with either HMPV83, rHMPV, ΔSH, ΔG, or ΔSH/G virus (0.1 PFU per cell), incubated for 10 days at 32°C in the presence of 5 μg of trypsin/ml, and harvested by scraping. The cell suspensions were adjusted to contain 50 mM HEPES (pH 7.5) and 0.1 M $MgSO_4$ and clarified by low-speed centrifugation at 2,000 rpm for 20 min at 4°C in a Beckman Coulter Allegra 6R centrifuge. The cell pellet in a small volume of medium was subjected to three cycles of freezing and thawing, the mixture was clarified, the supernatant fluids were combined, and the virus was pelleted by centrifugation at 7,000 rpm in a Beckman JLA rotor overnight at 4°C. The virus pellet was resuspended in OptiMEM containing 50 mM HEPES buffer and 0.1 M $MgSO_4$, layered on a 30-to-60% (wt/vol) discontinuous sucrose gradient, and centrifuged for 90 min at 26,000 rpm at 4°C in an SW28 rotor. The virus-containing band at the 30%-60% interface was collected and diluted in TEN (10 mM Tris-HCl [pH 7.4], 0.1 M NaCl, 1 mM EDTA), and virus particles were pelleted by centrifugation at 25,000 rpm in an SW28 rotor for 90 min at 4°C. Finally, the virus pellet was resuspended in TEN, and the protein yield of each preparation was quantified using the BCA protein assay kit (Pierce).

Protein electrophoresis and Western blot assay. Aliquots of sucrose-purified virus (10 μg for Coomassie blue staining or 1.5 μg for Western blot analysis) were reduced and denatured by heating at 99°C for 5 min and analyzed on a 4-to-20% polyacrylamide bis-Tris gel (NuPAGE Novex bis-Tris; Invitrogen). The separated proteins were stained with a Coomassie blue staining kit (Invitrogen) or electrotransferred onto a polyvinylidene difluoride membrane (Invitrolon; Invitrogen). The membrane was blocked for 2 h at room temperature in Western blocking reagent (Roche) and then incubated with a rabbit antiserum raised either against peptide G1-17, representing aa 1 to 17 of the HMPV83 G N terminus, against peptide G203-219, representing aa 203 to 219 of G (the C terminus), or against peptide SH82-96, representing aa 82 to 96 of SH. HMPV F was detected using a hyperimmune serum produced by immunization of hamsters with rHPIV1 expressing HMPV83 F from an added gene (22). Bound antibodies were visualized by binding with horseradish peroxidase-conjugated goat anti-rabbit or anti-hamster immunoglobulin G antibodies (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) followed by chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce). The Coomassie blue-stained gel and Western blot autoradiographies were scanned using a densitometer (Personal Densitometer SI; Molecular Dynamics) to quantify HMPV proteins.

Determination of replication and protective efficacy of ΔSH, ΔG, and ΔSH/G

viruses in hamsters. Groups of 6-week-old Golden Syrian hamsters were inoculated intranasally, under light methoxyflurane anesthesia, with 0.1 ml of L15 medium containing 5.7 $\log_{10}$ $TCID_{50}$ of HMPV83, rHMPV, ΔSH, or ΔSH/G virus or 5.2 $\log_{10}$ $TCID_{50}$ of rHMPV or ΔG virus. On days 3 and 5 postinfection, the lungs and nasal turbinates were harvested from six animals from each group, and the virus titer of individual specimens was quantified by serial dilution of tissue homogenates, as described above. To determine the level of immunogenicity and protective efficacy of the recombinants, additional hamsters in groups of six were infected with 5.7 $\log_{10}$ $TCID_{50}$ of rHMPV, ΔSH, or ΔSH/G virus or 5.2 $\log_{10}$ $TCID_{50}$ of ΔG virus in a 0.1-ml inoculum or with L15 medium diluent. Sera were collected 2 days prior to infection and 27 days postinfection. On day 28 postinfection, hamsters were challenged by intranasal administration of 5.7 $\log_{10}$ $TCID_{50}$ of HMPV83 in a 0.1-ml inoculum. Nasal turbinates and lungs were harvested 3 days later, and the titer of virus in each tissue homogenate was determined as described above.

HMPV serum neutralization assays. The titers of HMPV-neutralizing antibodies were determined using an endpoint dilution neutralization assay as described previously (22). Briefly, 75 μl of MEM containing approximately 240 $TCID_{50}$ of HMPV83 was mixed with an equal volume of MEM containing serial twofold dilutions of hamster serum that had been heat inactivated at 56°C for 30 min. The virus-antibody mixture was incubated at 37°C for 1 h, and 50 μl of this mixture was then transferred to each of two wells of a 96-well plate containing LLC-MK2 cells. After 1 h at 37°C the virus-antibody mixture was removed, and the monolayers were washed twice and incubated at 32°C for 8 days in OptiMEM supplemented with trypsin. Infected cultures were detected by immunoperoxidase staining with polyclonal rabbit anti-HMPV serum, as described previously (2). The neutralization titer was defined as the highest dilution of antibody at which half of the cultures were negative for infection.

RESULTS

Construction and recovery of rHMPV lacking the SH and/or G glycoproteins. The complete genes encoding the HMPV small hydrophobic SH and attachment glycoprotein G were deleted, singly or together, from a cloned cDNA encoding the HMPV antigenome (2). This was facilitated by the introduction of two new restriction sites, BsiWI and BsrGI, into the M2-SH and SH-G intergenic regions, respectively (Fig. 1). Cleavage at these two sites yields the same compatible overhang, GTAC, which also is compatible with a unique, naturally occurring Acc65I site in the G-L intergenic region. Thus, the SH and G genes could readily be deleted individually or in combination by the appropriate double digestion and ligation (Fig. 1) (see Materials and Methods). These gene deletions resulted in new intergenic regions of 163 nt (the M2-L intergenic region of ΔSH/G), 165 nt (the SH-L intergenic region of ΔSH), and 122 nt (the M2-G intergenic region of ΔG), which are intermediate in size between the naturally occurring G-L (190 nt) and SH-G (124 nt) intergenic regions (Fig. 1). For the genes in the vicinity of the deletions, each one that was preceded or followed in wild-type HMPV by either a short or long intergenic region was similar in the gene deletion mutants (e.g., G was always preceded by and followed by long intergenic regions, SH was always preceded by a short intergenic region and followed by a long one, and L was always preceded by a long intergenic region). The HMPV antigenomes expressed by the respective plasmids were 12,695 (ΔSH), 12,475 (ΔG), and 11,835 nt (ΔSH/G) in length, compared with the 13,335 nt for the parental rHMPV.

rHMPVs were readily recovered by transfection of BSR T7/5 cells with each respective full-length plasmid together with the four support plasmids encoding the HMPV N, P, M2-1, and L proteins (2, 3). The recombinant viruses were amplified by two passages in LLC-MK2 cells, and two-passage stocks were used in all experiments. Viral RNA from each gene deletion mutant was subjected to reverse transcription-PCR using primers specific to the end of the M2 gene and the beginning of the L gene. In each case, the product obtained was consistent with the expected deletion and was dependent on the reverse transcription step and, hence, was derived from RNA rather than from contaminating DNA (data not shown). Furthermore, each fragment was completely sequenced, confirming the expected structure.

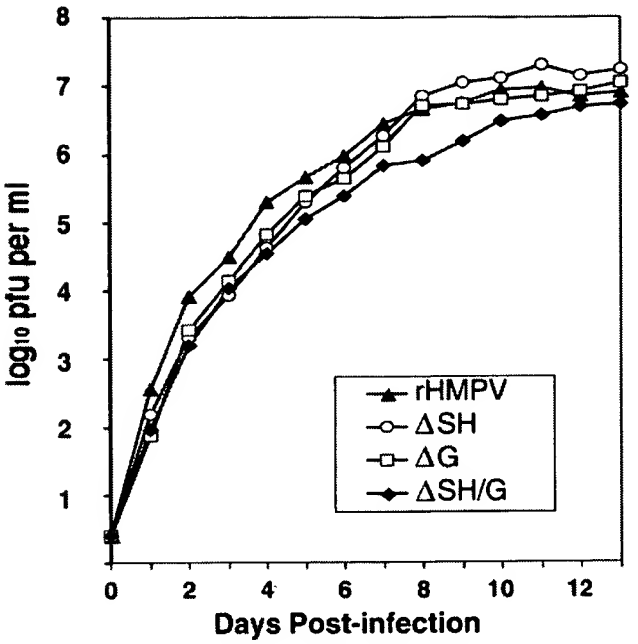

FIG. 2. Comparison of the multistep growth kinetics of the gene deletion mutants. LLC-MK2 cells were infected at an MOI of 0.01 PFU per cell with wild-type rHMPV (▲), ΔSH (○), ΔG (□), or ΔSH/G (◆). Supernatant aliquots (0.5 ml out of a total medium volume of 2 ml per well) were taken on the indicated days postinfection and replaced by an equivalent volume of fresh medium containing 5 μg of trypsin/ml. The samples were flash-frozen and analyzed later by plaque assay. Each time point was represented by two wells, and each virus titration was done in duplicate. Means are shown. The standard errors were calculated, but the bars are not shown because the errors were very small and the bars were obscured by the symbols.

Multicycle growth in vitro of the gene deletion mutants. The ΔSH, ΔG, and ΔSH/G viruses were compared to wild-type rHMPV with regard to the efficiency of multicycle replication in LLC-MK2 cells following infection with an input of 0.01 PFU per cell (Fig. 2). As previously shown, biologically derived HMPV83 and rHMPV replicated with similar efficiencies in vitro (2). rHMPV containing the deletion of a single gene, either SH or G, replicated as well as or somewhat more efficiently than rHMPV. The final titers reached by ΔSH and ΔG viruses were 1.7×10^7 and 1.1×10^7 PFU per ml, respectively, compared to 7.8×10^6 PFU per ml for rHMPV. Thus, these genes are not essential for replication in vitro, and the loss of the SH and G genes seems to marginally improve the viral replicative fitness in vitro, particularly in the case of the ΔSH virus. rHMPV lacking both SH and G replicated with an efficiency reduced 5.5-fold on day 8 postinfection compared to that of rHMPV, but the final titer reached by ΔSH/G was similar to that of rHMPV, 5.3×10^6 versus 7.8×10^6 PFU per ml.

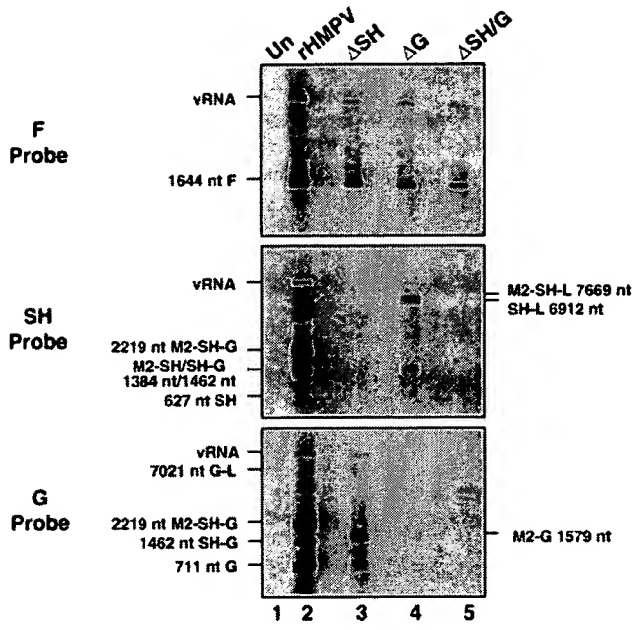

FIG. 3. Northern blot analysis of RNA expressed by the gene deletion mutants. LLC-MK2 cells were mock infected (lanes 1; Un) or infected at an MOI of 3 PFU per cell with wild-type rHMPV (lane 2), ΔSH (lane 3), ΔG (lane 4), or ΔSH/G (lane 5). Total intracellular RNA was isolated 72 h postinfection, electrophoresed on 1% agarose–formaldehyde gels, transferred to charged nylon membrane, and analyzed by hybridization with a double-stranded ^{32}P-labeled DNA probe specific to the F, SH, or G gene. The identities and the calculated sizes [exclusive of poly(A)] of individual mRNA species are indicated on the left for the naturally occurring species and on the right for new readthrough mRNAs created by the gene deletions. The bands marked vRNA contain both genome and antigenome.

Analysis of RNA synthesis by the gene deletion viruses. The synthesis of viral RNA and mRNA was analyzed by Northern blot hybridization performed on total RNA from HMPV-infected cells (Fig. 3). The blots were hybridized with double-stranded ^{32}P-labeled DNA probes generated by random priming from F, SH, or G gene cDNA fragments. Replica blots (data not shown) were hybridized with probes corresponding to the M2 and L genes in order to specifically identify each RNA species. For each virus, the F probe detected two bands: a major band corresponding to the 1644-nt [exclusive of poly(A)] F mRNA and a fainter band of the appropriate size to contain genome and antigenome (vRNA), which for the ΔSH, ΔG, and ΔSH/G viruses were shorter in length, reflecting the gene deletions. The Northern blotting performed with the F probe showed that there were reduced levels of vRNA as well as F mRNA for the gene deletion mutants, particularly for the ΔSH/G mutant. This was somewhat variable between different experiments performed with the same RNA preparations and likely reflected nonhomogenous RNA transfer in this series of Northern blot assays.

The Northern blotting performed with the SH and G probes confirmed the absence of the deleted genes in the genomes of the deletion mutants, evidenced by the lack of hybridization with vRNA as well as the absence of the corresponding mRNA. Interestingly, analysis with the SH-specific probe showed that wild-type rHMPV expressed a very low level of monocistronic SH mRNA, whereas there were strong bands corresponding to M2-SH, SH-G, and M2-SH-G readthrough mRNAs. The same observation was made with biologically derived wild-type HMPV83 (data not shown). This likely reflects inefficient transcriptional termination at the upstream and downstream gene end signals and will be investigated further in future work. With the G-specific probe, three major signals were observed with total RNA extracted from rHMPV-infected cells, corresponding to the 711-nt monocistronic G mRNA and the SH-G and M2-SH-G readthrough mRNAs. A very faint G-L readthrough mRNA also was detected. In addition, the SH- and G-specific probes hybridized to novel species that were unique to the deletion mutants and appeared to be new readthrough mRNAs that were consistent with the altered gene maps: M2-G for ΔSH virus and SH-L and M2-SH-L for ΔG virus.

Analysis of proteins in purified virions of the gene deletion viruses. Since the HMPV SH and G proteins are presumed to be structural proteins, by analogy to HRSV, it was of interest to compare purified virions of the gene deletion viruses and wild-type HMPV by gel electrophoresis, direct protein staining, and Western blot analysis. LLC-MK2 cells were infected with HMPV and, 10 days postinfection, the medium supernatants were harvested and viruses were concentrated and purified by sucrose gradient centrifugation. In one purification, we assayed the titer of infectious particles at different stages in the process and found that the total number of PFU was essentially undiminished by the purification process, suggesting that the particles remained largely intact. Ten micrograms of each sucrose-purified virus protein was separated on a 4 to 20% polyacrylamide gel and visualized by Coomassie blue staining (Fig. 4). The major protein bands visualized by staining were identified as the N, P, and M2-1 proteins by Western blot analysis of lysates of BSR T7/5 cells transfected with T7-driven plasmids expressing either N, P, or M2-1 and analysis with a rabbit antiserum against purified HMPV83 virions (data not shown). The SH, G, and F proteins could not be unequivocally detected, due to low abundance or inefficient staining with Coomassie blue, as is characteristic of glycoproteins. Overall, the pattern of viral proteins detected by direct staining was very similar for each of the gene deletion viruses (Fig. 4), with minor differences that appeared to represent experimental variability. Densitometer quantification of the N, P, M, and M2-1 proteins indicated that they were incorporated in approximately the same proportion in each of the different viruses (data not shown), and the sum of the four proteins was approximately the same for each virus (Fig. 4). This indicated that that deletion of the SH and/or G genes did not result in a dramatic change in virion composition or yield.

The presence of the SH and G proteins in virions was investigated by Western blot analysis with rabbit antisera raised against peptides representing an internal part of the SH ectodomain (SH82-96), the N terminus of G (G1-17), or the C terminus of G (G203-219) (Fig. 5). The electrophoretic patterns of these proteins had not been previously described. The species of the SH protein detected included a species of 23 kDa, an abundant species of 25 to 30 kDa, and a more-diffuse species of 80 to 220 kDa and even higher (Fig. 5A). These species were present in the appropriate viruses, namely, biologically derived HMPV83, rHMPV, and ΔG, and absent as

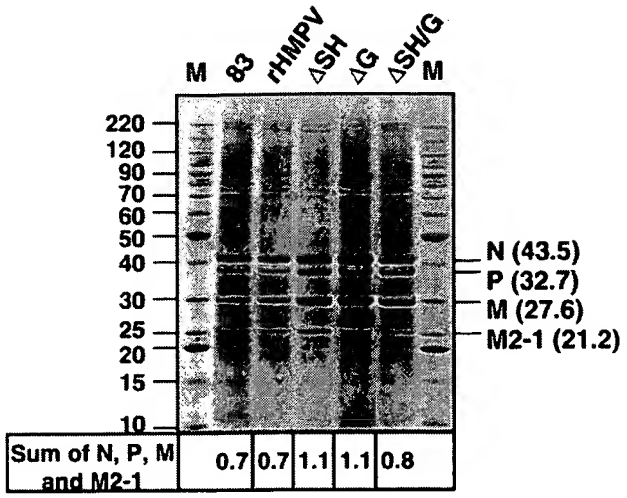

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified virions of wild-type HMPV and gene deletion mutants. Sucrose gradient-purified virions of wild-type biologically derived HMPV83 (83), wild-type rHMPV, ΔSH, ΔG, or ΔSH/G, as indicated, were analyzed on a 4-to-20% polyacrylamide gel, and proteins were visualized by Coomassie blue staining. The HMPV proteins are indicated on the right together with the molecular mass of the complete unmodified protein as calculated from the nucleotide sequence. Molecular markers (M) are shown, with molecular masses in kilodaltons indicated at the left. Underneath each lane is shown the sum of the N, P, M, and M2-1 bands as measured by densitometer, in arbitrary units.

expected in the ΔSH and ΔSH/G viruses. The 23-kDa species (designated SH0) was the appropriate size to be the complete nonglycosylated SH protein (calculated mass of 20.6 kDa), while the other two species (designated SHg1 and SHg2) presumably are glycosylated forms, by analogy to HRSV. Based on its electrophoretic difference relative to SH0, SHg1 might contain one or two N-linked carbohydrate side chains: the predicted sequence of the HMPV83 SH protein contains three potential Asn-X-Ser/Thr acceptor sites in the predicted ectodomain (1). Based on its high molecular mass and diffuse pattern, SHg2 might correspond to the polylactosaminoglycan-containing form that has been identified for HRSV. These three forms also were detected by Western blotting with a rabbit serum raised against peptide SH1-15 representing aa 1 to 15 of the HMPV83 SH N terminus (data not shown). The amount of SH protein in ΔG virions was consistently low compared to that in HMPV83 and rHMPV, amounting to 26% of that in the wild-type viruses (mean of three independent experiments). This was reminiscent of results with HRSV, where the incorporation of SH protein into virions of a G-deletion mutant was 25% that of wild type (25).

Western blot analysis with antiserum to peptide G203-219, representing the C terminus ectodomain, detected a single major species of 80 to 100 kDa (Gg), which presumably represents the full-length, mature glycosylated form of the protein (Fig. 5B). The calculated mass for the complete unmodified polypeptide moiety of G is 23.7 kDa, and the greater size of the virion-associated form suggests that it contains a high content of carbohydrate, comparable to that of HRSV. Surprisingly, using a serum raised against the G cytoplasmic tail (G N terminus G, G1-17), the G protein appeared as a ladder of bands, including ones of approximately 60, 50, 35, 20, and 13 kDa in addition to the complete 80- to 100-kDa form (Fig. 5C). The observation that these species reacted with antibodies raised against peptide G1-17 and not G203-219 indicated that they are C-terminally truncated species. These might arise due to proteolysis by the trypsin present in the medium, and it is noteworthy that the predicted ectodomain of the HMPV G protein is rich in lysine (six residues) and arginine (nine residues).

To detect HMPV F protein in purified virions, Western blots were incubated with hamster hyperimmune serum obtained by immunization with rHPIV1 expressing HMPV83 F as an added gene. Under nonreducing, nondenaturing conditions, similarly abundant amounts of F-specific signals were detected (Fig. 5D), spanning over a high-molecular-mass range of 120 to 220 kDa and higher. These might represent dimeric and trimeric forms of F. Additionally, a more discrete band was detected at a molecular mass of about 58 to 60 kDa, consistent with the expected molecular mass of the predicted disulfide-linked F1 and F2 subunits (or their uncleaved F_0 precursor). When analyzed under reducing conditions (Fig. 5E), F-specific signals were detected at a molecular mass of about 47 kDa, which would be the expected size of the F_1 subunit. This showed that the 58-to-60-kDa band as well as the high-molecular-mass putative F multimers detected under nonreduced conditions consist of disulfide-linked F_1 and F_2 rather than uncleaved F_0. Using the F-specific hyperimmune serum, we were not able to detect any low-molecular-weight protein that could be identified as F_2 (data not shown).

Replication, immunogenicity, and protective efficacy following intranasal administration to hamsters. Golden Syrian hamsters were infected intranasally either with 5.7 $\log_{10}$ $TCID_{50}$ of HMPV83, rHMPV, ΔSH, or ΔSH/G or with 5.2 $\log_{10}$ $TCID_{50}$ of rHMPV or ΔG (Table 1). The ΔG virus was administered at a lower dose because we wished to use the same low-passage stock that had been used for sequencing and most of the other analyses, and it was of lower titer. Thus, an additional group of animals were administered rHMPV in parallel at the same lower dose for direct comparison. Six animals from each group were sacrificed 3 and 5 days later, the nasal turbinates and lungs were harvested, and viral titers were determined (Table 1). Recombinantly derived rHMPV replicated in vivo with an efficiency similar to that of its biologically derived parent, HMPV83: the titer of rHMPV was 20- and 3-fold lower in the nasal turbinates and lungs, respectively, on day 3, but there was no significant difference on day 5. Thus, rHMPV appears to have wild-type-like growth properties in vivo as well as in vitro and represents a suitable starting point for developing attenuated derivatives as candidate vaccines. Also, the two different doses of rHMPV used in this study were similar in terms of the level of replication in the upper and lower respiratory tracts, allowing comparison between ΔG virus and the other viruses that were inoculated at a higher dose.

The replication of HMPV in vivo was not significantly reduced by deletion of the SH gene (Table 1). Indeed, the ΔSH virus replicated somewhat better than its wild-type counterpart, especially in the lungs, where a 20-fold increase was detected on day 5 postinfection compared to wild-type rHMPV. In contrast, the ΔG and ΔSH/G viruses were strongly attenuated. In the lungs, replication of the ΔG and ΔSH/G viruses was significantly reduced on day 3 (at least 40- and

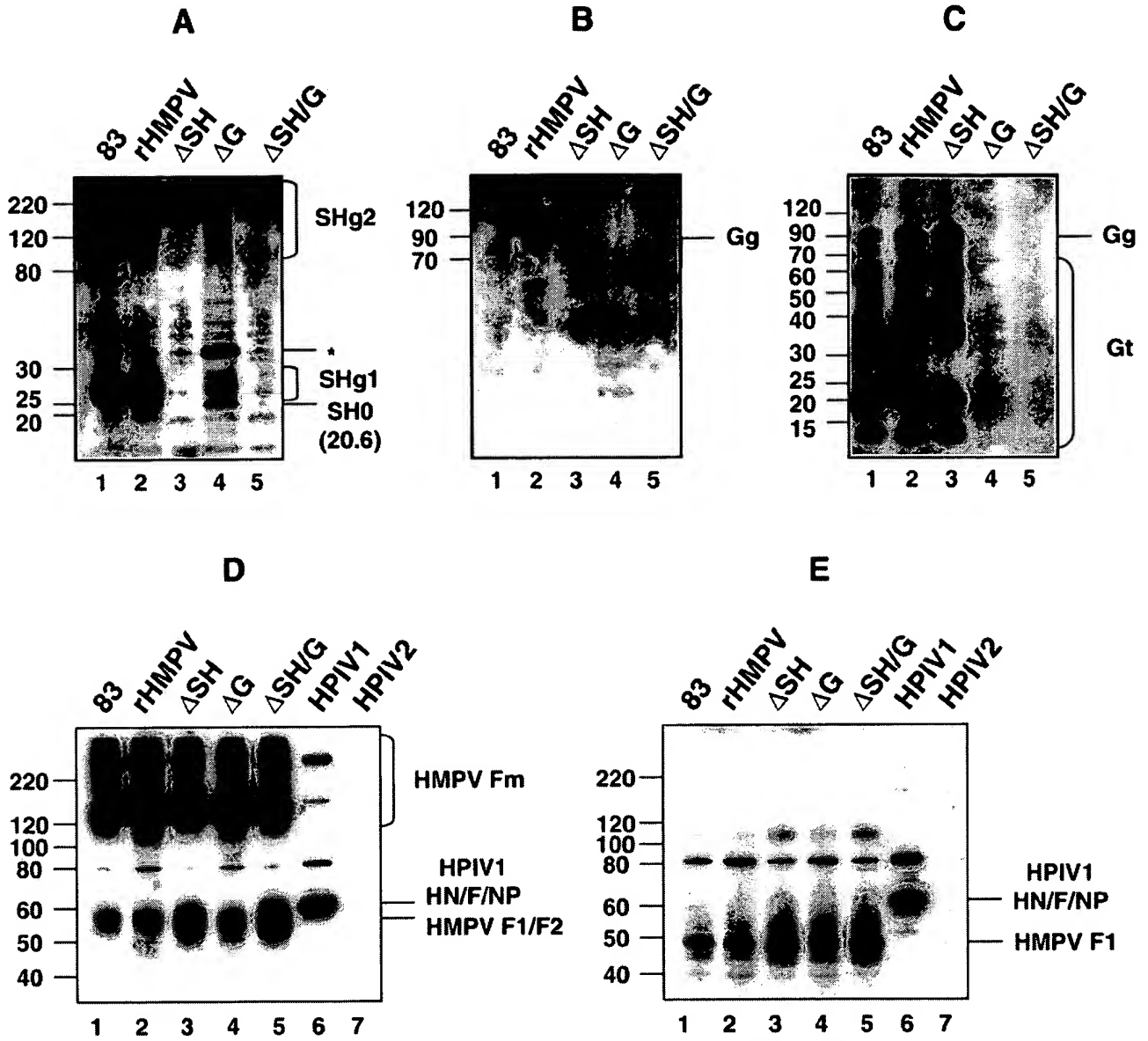

FIG. 5. Western blot analysis of the SH, G, and F proteins present in sucrose-purified HMPV virions. Purified virions of biologically derived wild-type HMPV83 (lanes 1, 83), wild-type rHMPV (lanes 2), ΔSH (lanes 3), ΔG (lanes 4), ΔSH/G (lanes 5), HPIV1 (lanes 6), or HPIV2 (lanes 7) were analyzed on 4-to-20% polyacrylamide gels under reducing and denaturing conditions (A, B, C, and E) or nonreducing and nondenaturing conditions (D). Following electrotransfer, membranes were incubated with an antiserum raised against peptide SH82-96, representing an internal part of the SH ectodomain (A); against peptide C203-219, representing the G C terminus (B); against peptide G1-17, representing the G N terminus (C); or against F protein, expressed by an HPIV1 vector (D and E). Bound antibodies were reacted with a peroxidase-conjugated goat anti-rabbit (A to C) or anti-hamster (D and E) immunoglobulin G and visualized by chemiluminescence. Tentative identifications of the various forms of the HMPV SH (A), G (B and C), and F (D and E) proteins are indicated on the right, as follows: SH0, unglycosylated form of SH; SHg1 and SHg2, putative glycosylated forms 1 and 2 of SH; Gg, glycosylated form of G; Gt, truncated forms of G; Fm, HMPV F multimers; F1-F2, disulfide linked HMPV F_1 and F_2 subunits; F1, HMPV F_1 subunit; HN, HPIV1 hemagglutinin neuraminidase; NP; HPIV1 nucleoprotein. An abundant cellular species that was variably present in the virions is indicated with an asterisk in panel A. The positions of molecular mass markers (in kilodaltons) are shown on the left.

100-fold, respectively) compared to the corresponding dose of wild-type rHMPV, but it was not significantly reduced on day 5 postinfection. Virus was recovered from the lungs of every animal on each day. In the upper respiratory tract, replication of the ΔG and ΔSH/G viruses was not detected in 33 and 17% of the animals, respectively, on day 3 but was detected in all animals on day 5. The level of replication of the ΔG and ΔSH/G viruses in the upper respiratory tract on both days was reduced more than 600- and 300-fold, respectively.

In order to evaluate immunogenicity and protective efficacy, the gene deletion viruses and their wild-type recombinant parent were administered intranasally to additional hamsters. Serum samples were taken 27 days later and analyzed in a neutralization assay (Table 2). The highly attenuated ΔG and

TABLE 1. Level of replication of gene deletion rHMPVs in the upper and lower respiratory tracts of hamsters

Virus[a]	Inoculum (log_{10} $TCID_{50}$/animal)	Day of harvest	Nasal turbinates			Lungs		
			% of animals with detectable virus	Mean titer (log_{10} $TCID_{50}$/g of tissue ± SE)[b] and statistical grouping[c]	Reduction of mean titer[d] (log_{10} $TCID_{50}$/g of tissue)	% of animals with detectable virus	Mean titer (log_{10} $TCID_{50}$/g of tissue ± SE)[b] and statistical grouping[c]	Reduction of mean titer[d] (log_{10} $TCID_{50}$/g of tissue)
HMPV83	5.7	3	100	6.4 ± 0.1, A		100	5.0 ± 0.1, AB	
rHMPV	5.7	3	100	5.1 ± 0.3, B		100	4.5 ± 0.2, A	
ΔSH	5.7	3	100	5.5 ± 0.3, B		100	5.3 ± 0.1, B	
ΔSH/G	5.7	3	83	2.2 ± 0.1,[e] C	2.9	100	2.5 ± 0.1, C	2.0
rHMPV	5.2	3	100	4.7 ± 0.1, D		100	3.8 ± 0.3, D	
ΔG	5.2	3	67	1.9 ± 0.1,[e] E	2.8	100	2.2 ± 0.1, E	1.6
HMPV83	5.7	5	100	5.6 ± 0.2, F		100	3.2 ± 0.1, F	
rHMPV	5.7	5	100	5.5 ± 0.2, F		100	3.0 ± 0.2, F	
ΔSH	5.7	5	100	5.6 ± 0.2, F		100	4.3 ± 0.1, G	
ΔSH/G	5.7	5	100	3.0 ± 0.2, G	2.5	100	3.0 ± 0.3, F	
rHMPV	5.2	5	100	5.4 ± 0.2, H		100	2.8 ± 0.3, H	
ΔG	5.2	5	100	2.6 ± 0.1, I	2.8	100	2.7 ± 0.2, H	0.1

[a] Hamsters in groups of six were administered 5.2 or 5.7 log_{10} $TCID_{50}$ (as indicated) of the indicated virus intranasally under light anesthesia on day zero.
[b] Animals were sacrified on day 3 and 5. Nasal turbinates and lungs were harvested and virus titers were determined. The lower limit of detection for virus in the upper and lower respiratory tracts was 1.5 log_{10} $TCID_{50}$/g of tissue. SE, standard error.
[c] Mean peak virus titers were assigned to statistically similar groups, separately for day 3 and day 5, by the Tukey-Kramer post hoc test for animals inoculated with 5.7 log_{10} $TCID_{50}$ (groups A to C, and F and G) or by Student's unpaired t test for animals inoculated with 5.2 log_{10} $TCID_{50}$ (groups D and E, H and I). Values within a column that share a common letter are not significantly different, whereas those that do not are significantly different ($P < 0.05$).
[d] Reduction of mean titer compared to the respective dose of rHMPV.
[e] The value of 1.5 log_{10} $TCID_{50}$/g of tissue was assigned to nonshedding animals to calculate the mean titer.

ΔSH/G viruses induced high titers of HMPV-neutralizing serum antibodies, which were less than sixfold reduced compared to those induced by rHMPV. On day 28 postimmunization, the animals were challenged intranasally with wild-type HMPV83, and the animals were sacrificed 3 days later. Nasal turbinates and lungs were harvested, and virus titers were determined by limiting dilution (Table 2). Challenge virus could not be detected in the nasal turbinates or lungs of any of the animals that had previously been infected with the rHMPV or ΔSH viruses. The animals that had been infected with the ΔG or ΔSH/G virus also were protected from challenge virus replication in the lungs. In the nasal turbinates, only 40% of the animals that had been immunized with ΔSH/G had detectable challenge virus, and the mean titer of challenge virus was reduced more than 3,000-fold. Challenge virus was detected in the nasal turbinates of all of the animals that had been immunized with the ΔG virus, but the mean titer was reduced by more than 300-fold compared to challenge virus replication in the mock-immunized group. Thus, despite their strongly attenuated nature, the ΔG and ΔSH/G viruses were immunogenic and highly protective against HMPV challenge.

DISCUSSION

An HMPV reverse genetic system was used to generate rHMPVs from antigenomic cDNAs in which the putative SH and G open reading frames, each with their surrounding set of gene start and gene end signals, were deleted singly or to-

TABLE 2. Immunogenicity and protective efficiency of gene deletion rHMPVs in hamsters

Immunizing virus[a]	Inoculum (log_{10} $TCID_{50}$/animal)	Mean serum neutralizing antibody titer (log_2 ± SE)[b]		Challenge virus replication on day 3 postchallenge			
				Nasal turbinates		Lungs	
		Preimmunization	27 days postimmunization	% with detectable challenge virus	Mean titer (log_{10} $TCID_{50}$/g of tissue ± SE)[c]	% with detectable challenge virus	Mean titer (log_{10} $TCID_{50}$/g of tissue ± SE)[c]
Mock		<3.3 ± 0.0	<3.3 ± 0.0	100	6.5 ± 0.1	100	4.8 ± 0.3
rHMPV	5.7	<3.3 ± 0.0	10.0 ± 0.2	0	≤1.5 ± 0.0	0	≤1.5 ± 0.0
ΔSH	5.7	<3.3 ± 0.0	10.4 ± 0.2	0	≤1.5 ± 0.0	0	≤1.5 ± 0.0
ΔSH/G	5.7	<3.3 ± 0.0	7.6 ± 0.3	40	3.0 ± 0.8[d]	0	≤1.5 ± 0.0
ΔG	5.2	<3.3 ± 0.0	7.5 ± 0.5	100	4.0 ± 0.5	0	≤1.5 ± 0.0

[a] Hamsters in groups of six (or five for ΔSH/G) were immunized by intranasal infection with 5.2 or 5.7 log_{10} $TCID_{50}$ as indicated of the indicated virus or mock infected with L15 medium (mock).
[b] Sera were collected 2 days before and 27 days following the first infection, and the neutralizing antibody titer against HMPV83 was determined. The preinfection anti-HMPV serum titers were <3.3 (reciprocal log_2) for all animals in the study.
[c] On day 28, hamsters from each group were challenged intranasally with 5.7 log_{10} $TCID_{50}$ of HMPV83. Nasal turbinates and lungs were harvested 3 days later, and the virus titers were determined on LLC-MK2 cells.
[d] The value of 1.5 log_{10} $TCID_{50}$/g of tissue (the lower limit of detection for virus) was assigned to nonshedding animals to calculate the mean titer.

gether. In each case, infectious virus was readily recovered. We then evaluated the kinetics and efficiency of growth in vitro of the deletion mutants and their replication, immunogenicity, and protective efficacy in experimental animals.

The amino acid sequence of the HMPV G protein deduced from the nucleotide sequence of its gene suggests that it is a type II mucin-like glycosylated protein with similarities to the G protein of HRSV, representing a separate genus in subfamily *Pneumovirinae*. The size of the HMPV G protein determined here by Western blot analysis suggested that it is heavily glycosylated, approximately to the same extent as HRSV G. The HMPV G protein detected in purified virions with antibodies specific to its C terminus had a high molecular mass of 80 to 100 kDa, compared to a value of 23.7 kDa calculated for its unmodified polypeptide backbone deduced from the gene sequence. Western blot analysis of HMPV virions with antibodies specific to the N terminus of G detected a ladder of C-terminally truncated forms of G that might represent proteolytic degradation products due to the trypsin in the medium. The finding that C-terminally truncated forms purify with virions, whereas no N-terminally truncated fragments were found, is consistent with the idea that HMPV G indeed is anchored in the membrane by its N-terminally proximal hydrophobic domain. The apparent sensitivity of HMPV G to proteolysis at multiple sites throughout its backbone would be consistent with an exposed, extended, nonglobular structure.

The SH protein of HMPV is substantially longer (179 versus 64 aa) than that of HRSV, the pneumovirus SH protein that has been characterized in the greatest detail. However, the HMPV SH protein has similar characteristics to that of HRSV, including a high percentage of threonine and serine residues and a similar hydrophilicity profile (1, 29). Western blot analysis of the SH protein present in HMPV virions provided evidence of multiple forms that appeared to correspond to some of those of HRSV. These include a candidate to be the complete unglycosylated SH protein (SH0, 23 kDa), a candidate to be the N-glycosylated SH protein (SHg1, 25 to 30 kDa), and a candidate to be a more extensively glycosylated form (SHg2, 80 to 220 kDa and higher). Moreover, in the absence of the G protein, the HMPV SH protein was found to be less efficiently incorporated in virion particles (26% of that in the wild-type virus), which also was seen for the HRSV G deletion mutant (25). Recent data showed that the HRSV F, G, and SH glycoproteins can form an oligomeric complex within the HRSV-infected cell (7). It may be that, within this proposed complex, interactions between G and SH occur such that the absence of G results in a less efficient incorporation of SH at budding sites. To date, the function of the pneumovirus SH protein remains unclear, but the ability to delete HMPV SH with no detrimental effect on replication in vitro or in vivo indicates that it does not play an irreplaceable role in attachment or entry. The HRSV SH protein appears to assemble into homopentamers, and a channel-like activity is suggested by the increased membrane permeability to low-mass compounds observed when the SH protein is expressed in bacteria (6, 14, 20). Thus, HRSV SH protein appears to have characteristics of a viroporin, which modifies membrane permeability (8). By analogy with HRSV, the HMPV SH protein also is a candidate to be a viroporin, which would be the longest known member to date. However, its permeabilization capacity remains to be proven.

In cell culture, rHMPV viruses containing the deletion of a single gene, either SH or G, replicated as well as or somewhat more efficiently than the wild-type virus, indicating that these genes are not essential for replication in vitro. The loss of SH in particular seemed to improve the replicative fitness. This may reflect a growth advantage due to a shorter length of the genome and/or loss of a transcriptional unit increasing the expression of the downstream gene. It also is possible that the absence of the SH or G protein might somehow improve growth. For example, it was found for HRSV that deletion of the SH gene enhanced the rate of virion entry, cell-to-cell fusion, and plaque size, suggesting an inhibitory effect of the SH protein on F protein activity (25). Effects due to ablating expression of the SH and G proteins versus changing the genome length and number of genes will be evaluated in the future by constructing ΔSH and ΔG viruses in which the deleted gene is replaced by a stuffer that is identical with regard to intergenic and gene start and gene end signals and which contains a foreign sequence of identical size that does not encode a protein. For the present, we are interested in the expeditious development and characterization of possible vaccine candidate viruses, where a stuffer gene would be unacceptable for further development for clinical testing. The present results show that the HMPV F protein alone is sufficient to mediate attachment and fusion in the absence of the other surface proteins. If HMPV G protein has a functional role for attachment, as postulated, it would not be the sole attachment protein. Moreover, HMPV G and SH are not required for the efficient assembly or release of progeny virus, as also is the case for bovine and human RSV (12, 13).

When inoculated intranasally into hamsters, recombinantly derived HMPV replicated less efficiently on day 3, but not on day 5 postinfection, compared to its biologically derived HMPV83 parent. The recombinant virus was designed and confirmed to be identical to the consensus sequence of the biological virus, apart from four nucleotide substitutions involved in creating the NheI marker site, but the slight difference might be explained by the fact that the clinical isolate HMPV83 has not been plaque purified and thus might contain minority variants that contribute disproportionately to growth in vivo. However, the difference was not great, and rHMPV appeared to have wild-type-like growth properties in vivo as well as in vitro (2) and thus represents a suitable starting point for developing attenuated derivatives as candidate vaccines. The replication of the ΔSH virus in hamsters was not significantly reduced in the upper respiratory tract and, surprisingly, was increased 20-fold in the lower respiratory tract on day 5 postinfection. Moreover, it was equivalent to its wild-type parent in the ability to induce neutralizing serum antibodies and to confer complete protection against a subsequent wild-type HMPV infection. Therefore, the HMPV SH gene appeared to be completely dispensable for growth in vivo in hamsters. This may represent an unusual situation in which deletion of a viral gene actually improves growth, but this will need to be verified in additional experiments, including with a model closer to the natural host. This clearly differs from the situation with HRSV, where virus that lacked the SH gene was reduced 10-fold in replication in the upper respiratory tract of mice (4) and was

moderately attenuated in the respiratory tract of chimpanzees (33).

The HMPV mutants lacking G, ΔG and ΔSH/G, were reduced in replication by at least 600- and 40-fold in the upper and lower respiratory tracts of hamsters, respectively, compared to that with rHMPV on day 3 postinfection. In this experiment, the ΔG and ΔSH/G viruses were recovered 3 days postinfection from the upper respiratory tract of 67 and 83% of the animals, respectively, and from the lower respiratory tract of 100% of the animals. Since each animal shed virus on day 5 postinfection, both ΔG and ΔSH/G unambiguously replicated in all infected animals. This is somewhat different from the situation with the G-deletion rHRSV mutant in mice, where virus was not recovered from the upper respiratory tract and a very low titer was recovered from the lower respiratory tract of 60% of the animals, indicating that rHRSV ΔG is overattenuated in mice and might not replicate at all since the trace amount that was recovered might have represented input virus (28). Similarly, it was not clear that the BRSV ΔG deletion mutant was able to replicate in the bovine respiratory tract (21). Thus, HMPV provides a clear demonstration that the F protein alone as a virion surface protein is sufficient for replication in vivo, albeit at a reduced level.

Immunization of animals with either the ΔG or ΔSH/G virus induced a high titer of HMPV-neutralizing serum antibodies, even though these viruses were highly attenuated. Importantly, the animals were completely protected in the lungs against a subsequent HMPV83 challenge, and only a moderate level of challenge virus replication was detected in the nasal turbinates. Thus, despite their strongly attenuated phenotype in vivo, the ΔG and ΔSH/G viruses were immunogenic and highly protective against HMPV challenge and represent promising vaccine candidates. It was interesting that deletion of the G gene yielded a promising, replication-competent vaccine candidate, since the corresponding deletion in HRSV yielded a virus that was overattenuated in mice and humans (13, 28). An rBRSV lacking the G gene may not have been competent for replication in vivo but still was able to protect calves, its natural host, against a subsequent infection (21), but our experience with attenuated human respiratory viruses is that replication is necessary in order for the virus to be satisfactorily immunogenic. With HRSV, deletion of the G protein from vaccine virus also is considered undesirable, because G is one of the two major neutralization and protective viral antigens. However, the contribution of HMPV G to neutralization and protective efficacy may be relatively minor (unpublished data). Furthermore, the HMPV F protein alone expressed from recombinant vectors induced a high level of neutralizing antibodies and protective immunity against homologous and heterologous HMPV strains (22, 24). Thus, the ΔG and ΔSH/G viruses represent vaccine candidates with promising levels of attenuation and protective efficacy that can be studied further in nonhuman primates such as African green monkeys and should be prepared for clinical evaluation.

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Animal Models of Respiratory Syncytial Virus Infection

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Over the past two decades, animal models of respiratory syncytial virus (RSV) infection have been developed using primates, cotton rats, mice, calves, guinea pigs, ferrets, and hamsters. Use of these models has shed light on the mechanisms of vaccine-enhanced disease seen in clinical trials of a formalin-inactivated RSV vaccine and has provided a means for testing efficacy and safety of candidate prophylactic and therapeutic strategies. The development of multiple animal models has coincided with the realization that RSV disease in humans is a multifaceted disease whose clinical manifestations and sequelae depend upon age, genetic makeup, immunologic status, and concurrent disease within subpopulations. There is no single human subpopulation in whom all forms of RSV disease manifest, nor is there a single animal model that duplicates all forms of RSV disease. The choice of an experimental model will be governed by the specific manifestation of disease to be studied.

Respiratory syncytial virus (RSV) has been enigmatic since its discovery >40 years ago. Initially isolated from chimpanzees during an epizootic of upper respiratory tract disease [1], RSV was subsequently found to be the most important cause of infectious pulmonary disease in human infants. More recently, RSV has been shown to be a major pathogen in immunosuppressed and elderly individuals.

RSV is a member of the family Paramyxoviridae, subfamily Pneumovirinae, and has a nonsegmented negative stranded genome of 15,222 nucleotides (strain A2) [2]. The virion consists of a nucleocapsid within a lipid envelope, and the virions are irregular in size and shape. There are two major glycoproteins expressed on the virion surface: the fusion protein and the attachment protein. A small hydrophobic protein is also expressed on the virion surface. Two major antigenic groups (A and B) have been described; these groups are distinguished primarily by dissimilarities in the attachment protein (1%–7% relatedness). Other related viruses include bovine RSV, ovine RSV, caprine RSV, pneumonia virus of mice, and turkey rhinotracheitis virus. RSV is structurally and functionally similar to parainfluenza viruses, although there is little antigenic or sequence relationship. RSV is much more distantly related to the family Orthomyxoviridae (influenza virus), which have a segmented genome.

In one prospective study of children [3], the rate of RSV infection was 68.8 cases per 100 children during the first year of life and 82.6 cases per 100 children during the second year of life. By 24 months of age, all of the children had been infected at least once, and one-half had had two infections. It is estimated that the incubation period for RSV infection is 4 or 5 days, and transmission probably occurs via fomites rather than aerosols. There is no viremia.

Pulmonary involvement occurs in 25%–40% of initial infections but is uncommon in subsequent infections, except those in recent bone marrow transplant recipients or elderly patients. Virus may be shed as long as 20 days. There is no evidence of persistent infection in immunocompetent individuals. Premature infants or those with cyanotic congenital heart disease or bronchopulmonary dysplasia are especially likely to develop severe pulmonary infections with RSV. The immune response of infected individuals does not appear to be protective for longer than a few months; whether the temporary protection is due to antibody or to cellular immunity is unclear.

The development of the first vaccine (employing the same technology—formalin inactivation—as the highly successful Salk vaccine) and subsequent vaccination of human children ended with disastrous results when natural RSV infection developed in those children (up to 80% of children were hospitalized, and two children died) [4]. These results have overshadowed efforts to develop an RSV vaccine for three decades and still stand as a barrier to licensure.

Interpretation of the vaccine trials was severely hampered by the lack of a small animal model in which vaccine-enhanced disease could be reproduced and studied experimentally. In the absence of such a model, the vaccine's sponsors—observing that the moderate serum antibody responses in the vaccinees provided no apparent protection—concluded that serum antibody to RSV actually enhanced disease rather than protecting against it [5]. This hypothesis gained widespread acceptance for nearly 15 years until the advent of small animal models, whereupon studies in those models (particularly the cotton rat model) disproved the hypothesis and established the foundation for antibody-based immunoprophylaxis.

The development of models of RSV infection and disease, particularly in the cotton rat [6, 7] and the inbred mouse [8], marked turning points in research efforts to prevent RSV disease and to understand the mechanisms of vaccine-enhanced disease. Studies in the cotton rat model showed that serum IgG

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Table 1. Criteria to consider when developing an animal model of respiratory syncytial virus infection.

Genetics	The wide divergence of patterns of RSV disease among different human subpopulations [16] suggests a strong connection between host genotype and disease phenotype that may influence the choice of animal models.
Availability	Some types of studies require specialized host strains (inbred, transgenic, or knockout) and specific reagents for identification and quantitation of immunoglobulins, immune cells, and cytokines.
Husbandry	Practical issues include maintenance costs, ease of handling, anesthesia, surgery, dosing, and tissue sampling.
Numbers	Statistically significant numbers of animals per study are essential, and the use of one animal [17] or of historical rather than concurrent controls [18–21] may lead to statistically insignificant and potentially misleading data.
Dosage	An excessive dose of challenge virus may either obscure an otherwise effective prophylactic or therapeutic approach [22] or produce experimental disease that is not analogous to its human counterpart.

NOTE. RSV = respiratory syncytial virus.

with RSV-neutralizing activity (RSVIg) could prevent pulmonary infection and attenuate nasal infection [9–11], and these studies established the rationale for clinical trials of RSVIg prophylaxis with plasma-derived IgG for infants at high-risk [12, 13]. These trials, in turn, led to the licensure of the first RSV preventive agent, RespiGam (MedImmune, Inc., Gaithersburg, MD), in 1996.

At the same time, results obtained with animal models challenged prevailing wisdom concerning RSVIg; they provided the foundation for dissecting the mechanisms of vaccine-enhanced disease [14]. These mechanisms are complex, involve many arms of the immune response, and are not restricted to formalin-inactivated (FI) RSV formulations [15]. As it becomes apparent that various types of formulations, in addition to FI RSV vaccine, have the potential of stimulating an inappropriate immune response, the issue of vaccine safety becomes increasingly important. Although this issue will not be resolved completely until an RSV vaccine has been tested in seronegative human infants, the decision to proceed to that stage will be based in large measure upon studies in animal models. Several pragmatic issues relating to animal modeling in general are listed in table 1. We now describe the most significant animal models of RSV infection and disease, emphasizing the relative strengths and weaknesses of each model without attempting to summarize all of the experimental data derived from their use.

Chimpanzee

Although no other laboratory animal approaches the genetic relatedness of chimpanzees to humans, practical and biological considerations severely limit the utility of chimpanzees in RSV research and perhaps cast doubts upon the relevance of published experimental studies. Chimpanzees are scarce, extremely expensive, and available in the United States only through primate breeding programs. The already small numbers of animals available to investigators will be reduced even further due to a recent reduction in funding for the breeding programs, raising the purchase cost even further. Current costs of leasing and caring for chimpanzees are also extremely high. Maintenance costs are further inflated because terminal experimentation in chimpanzees is generally not permissible, and thus the investigator must either maintain the animals for the duration of their natural lives or trade or sell them to other investigators for other purposes.

The net effect of these expenses has been that only one laboratory (Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health) has published studies of RSV infection in chimpanzees. Primary RSV infection was initially described in only four animals [23], in whom the study of infection was restricted to nasal tissues. Subsequent studies have been limited to four or fewer animals per group, and studies have often used historical rather than concurrent controls [17–21, 24]. The genetic heterogeneity to be expected among outbred animals and the statistical insignificance of data from so limited a number of observations raise concerns about the scientific validity of these experiments. Furthermore, the scarcity of chimpanzees precludes verification of published observations by other laboratories.

Biological considerations also suggest that the utility of the chimpanzee model is limited. Although advocates of the model emphasize the dramatic rhinorrhea accompanying experimental infection, no studies have documented pulmonary disease following primary infection [18]. Furthermore, no evidence exists that FI RSV vaccine can produce enhanced disease in the chimpanzee, thus raising questions about the relevance of the chimpanzee as a model of vaccine safety.

Other Primates

Experimental RSV infection has been described in the owl monkey [25, 26], rhesus monkey [23], African green monkey [27], cebus monkey [28], squirrel monkey [23], bonnet monkey [29], and baboon [30]. Purchase and maintenance costs for all of these species (in the United States), although considerably lower than those for the chimpanzee, are still high and tend to result in the use of statistically insignificant numbers of animals. Unlike chimpanzees, all of these species may be used in terminal experimentation, thus allowing more detailed virological and histological studies of pulmonary RSV disease.

Although these species all are closer genetically to humans than are rodents, it has yet to be demonstrated that their use provides data of greater or even equal relevance to the understanding, prevention, and treatment of human disease. Lack of

inbreeding in any of these species limits immunologic studies, and relative unrelatedness to humans (compared with the chimpanzee) has meant that few human immunologic reagents cross-react. None of these species has been shown to develop either clinical or radiological signs of pulmonary RSV disease, and pulmonary infection has been documented (though not well-characterized) only in the owl monkey. Owl monkeys and baboons develop mild rhinorrhea, whereas the other species show no signs of nasal disease.

The most useful of these species may be the African green monkey because the enhanced pulmonary disease it develops subsequent to immunization with FI RSV vaccine [27] is histologically similar to that seen in humans who died after vaccination [4]. However, the large number of animals that would be necessary to characterize fully the enhanced disease, the lack of inbreeding, and the scarcity of immunologic reagents suggest that African green monkeys will be, at best, a secondary model of vaccine-enhanced disease and vaccine safety.

Cotton Rat

RSV infection in the cotton rat was first demonstrated by Dreizin and co-workers [6], who described the kinetics of viral replication and histological changes in the rats' lungs. The cotton rat remains uniformly susceptible to pulmonary infection through adulthood, thus establishing it as a useful model for long-term studies [7]. In comparison with the mouse, the cotton rat is 100-fold more permissive (per input dose of virus) and more responsive immunologically, developing titers of serum antibody that are 10-fold or more higher [8, 31]. In addition to its greater permissiveness, the cotton rat develops vaccine-enhanced pulmonary disease that appears to parallel that in humans and other primates [14], whereas the mouse does not [32]. The candidate vaccines of Wyeth-Lederle Vaccines and Pediatrics (Pearl River, NY) [33], Upjohn (Kalamazoo, MI) [34], Pasteur Mérieux Connaught (North York, Ontario, Canada) [35], SmithKline Beecham Biologicals (Rixensart, Belgium) [36], and Institut De Recherche Pierre Fabre (Boulogne, France) [37] have been tested extensively in the cotton rat, and it currently serves as the primary model for the determination of vaccine safety.

Studies in the cotton rat showed that ribavirin (Virazole, ICN Pharmaceuticals, Costa Mesa, CA) effected a modest reduction in pulmonary virus (about 10-fold), but no histological data that might have given clues to the drug's ability to reverse the disease process were provided [38]. Subsequent studies in our laboratory failed to demonstrate an ameliorating effect on histopathologic changes [39], and recent clinical studies showing marginal or undetectable clinical benefit [40–42] are consistent with the data for the cotton rat. Studies with the cotton rat model also showed that serum neutralizing antibody was highly effective in preventing pulmonary infection [10, 11]. Subsequent clinical trials confirmed this observation [12, 13] and formed the basis of licensure of RSVIg as a preventive agent in 1996. Therapeutic studies in the cotton rat [9, 43] served as the basis for clinical trials of aerosolized [44] and intravenous [45] IgG treatment of hospitalized infants with RSV disease. Although IgG treatment reduced titers of virus, none of these studies showed a significant effect on clinical outcome. Our more recent work examining a combined antiviral/antiinflammatory approach [46] suggests that modulation of lung inflammation, in addition to clearance of virus, will be required for rapid reversal of clinical disease; clinical trials are currently being planned to test this approach.

Although there are several inherent advantages of the cotton rat model, there have been factors that have limited its use. Despite the fact that inbred cotton rats are now available commercially (Virion Systems, Rockville, MD), one major factor still limiting their use is the lack of reagents for characterization and quantitation of immunoglobulins, complement and other plasma proteins, cell surface antigens, and cytokines. Another disadvantage of the cotton rat is that, unlike the mouse, there are no congenic, transgenic, or knockout strains. The availability of such strains of mice and the unlikelihood of similar strains of cotton rats being developed suggest that RSV studies requiring such resources will continue to be done in the mouse.

Mouse

Shortly after RSV was discovered, Coates and Chanock [47] examined several species of laboratory animals to determine if any were permissive for pulmonary viral infection. Among the animals were four strains of inbred mice (DBA/2, BALB/c, AKR, and C3H). None of these mice developed CF antibody, and only one strain (AKR) developed neutralizing antibody; no attempt to recover infectious virus from the lungs was reported. Prince and co-workers [8] found that each of 20 inbred strains, including the four tested in the earlier report, was permissive for RSV infection in the lungs and nose. Levels of viral replication varied by two orders of magnitude from the least permissive (CBA/CaHN) to the most permissive (DBA/2N) strains, yet even the most permissive strain was about 100-fold less sensitive than the cotton rat [7].

The mouse model has several advantages over all other species, including a vast array of inbred, congenic, transgenic, and knockout strains; an unmatched library of specific reagents allowing identification and quantitation of cell types, immunoglobulins, cytokines, and other antigens; and relatively low purchase and maintenance costs. Although no prophylactic or therapeutic formulations have yet been licensed on the basis of studies in mice, many insights into the immunology of RSV disease have emerged that could not yet have been obtained from other models (for reviews of such studies, see [48, 49]). Of particular interest are studies describing different cytokine profiles in animals undergoing various types of immunization, including that with FI RSV vaccine [50]. However, although such profiles would be of great value in defining the parameters of safe vaccines, distinctly different cell types are obtained

from different inbred strains with use of bronchoalveolar lavage [51], and such differences (combined with the histological dissimilarity of vaccine-enhanced disease in the mouse and human [32]) have the potential of confusing rather than clarifying the pathogenesis of FI RSV vaccine–enhanced disease. Further research on FI RSV immunization of other inbred strains may clarify this issue.

Calf

Thirteen years after the discovery of RSV as a human pathogen, a related virus was recovered from cattle with epidemic respiratory tract disease [52]. Subsequent studies showed that bovine RSV was a ubiquitous pathogen of cattle throughout the world [53]. The diseases caused by RSV and bovine RSV have several common characteristics, suggesting that the calf may be a useful model of human RSV disease. For example, both viruses cause acute disease that is limited to the respiratory tract, induce an incomplete immune response permitting repeated infections throughout life, cause epidemic disease clustered during winter months, are attenuated by high levels of maternally derived antibody, and cause severe pulmonary disease primarily in neonates [53]. The calf model of bovine RSV infection, however, has many of the same practical drawbacks of primate models: the cost of purchasing and maintaining animals, lack of inbred strains, and lack of specific reagents.

Two characteristics of clinical disease caused by bovine RSV in calves raise concerns about the usefulness of the calf model. First, bovine RSV commonly causes fever, while human RSV does not [54]. It is not known whether this circumstance is due to differences in the viruses or in host responses. Second, bacterial (particularly pasteurella and haemophilus [55]) and mycoplasmal [56] coinfections are common complications of bovine RSV infection, whereas such coinfections with human RSV have rarely been identified.

Recent studies have shown that calves immunized with FI bovine RSV vaccine and then challenged intranasally with live homologous virus develop enhanced pulmonary disease similar to that seen in humans, African green monkeys, and cotton rats [57]. Although a model employing a viral pathogen in its natural host might have an advantage over a human pathogen in an unnatural host, there are several other factors to consider that might dampen enthusiasm for a calf model of human RSV infection. The limited homologies of amino acids in several RSV and bovine RSV proteins and the greater difficulty in propagating and titrating bovine RSV in vitro underscore the fact that these viruses, while related, are not identical. It is likely that the most sound approach to animal modeling would include the human virus in the most permissive experimental host plus a nonhuman but related virus in its natural host.

Guinea Pig

Coates and Chanock [47] reported that RSV infection in the guinea pig caused a moderate neutralizing antibody response, but no attempt to quantitate viral replication in the lungs or nose was reported. Indeed, no systematic description of viral replication has yet been published. The only study to quantitate infectious virus in the lungs [58] reported a single time point (6 days after infection) and a very low titer ($10^{3.2}$ pfu/g).

The chief advantage of the guinea pig model derives from the extensive knowledge of airway physiology, particularly reactive airway disease, in this species. A causal relationship between RSV infection early in life and subsequent development of asthma has been suggested for many years, but this relationship has not been proven. Heightened responsiveness to acetylcholine was observed 7 days after RSV infection in guinea pigs but not 14 days after infection [59], suggesting either that the guinea pig is not a suitable model or that other noncholinergic effectors of airway hyperreactivity might be modulated by RSV. Recent reports of the persistence of viral genome and protein, but not infectious virus, for up to 60 days provide an intriguing backdrop for further studies [60, 61].

The chief disadvantages of the guinea pig model are its apparent limited permissiveness for RSV infection, the general unavailability of inbred strains, and the scarcity of immunologic reagents.

Ferret

RSV replicates in high titers in the nasal tissues of ferrets of all ages [47], but the virus replicates in the lungs only of infant animals [62]. Although the rapidly decreasing permissiveness of the lung severely limits the utility of ferrets, it provides an intriguing model for the age dependence of severe RSV disease in humans (in whom the severity of primary pulmonary disease is inversely proportional to age); nasal disease in humans does not vary with age [63]. Subsequent studies of RSV infection in ferret lung tissue and monolayer cultures confirmed in vivo findings, thereby suggesting a local, nonimmunologic mechanism [64]. Despite the fact that the ferret model has disadvantages similar to those of the guinea pig and primate models (lack of inbred strains and immunologic reagents), it is the only model in which age-dependent pulmonary infection has been described and thus offers a potential tool for dissecting the mechanisms of age-dependent disease in humans.

Hamster

The only other animal for which quantitative virological studies have been reported is the Syrian hamster [65]. Although RSV replicated in both the lungs and nose, the relative permissiveness of the hamster was equivalent to that of the mouse and was ~100-fold less than that of the cotton rat. No follow-up reports have extended the quantitative studies of Wright et al. [65], and no studies describing RSV infection in hamsters have been published since 1983.

Summary

No single animal model has been shown to duplicate all aspects of primary RSV disease in the human infant at normal risk, much less in the increasing number of subpopulations for whom RSV poses threats ranging from inconvenience to death. Thus, there is no single answer to the question, "What is the best animal model of RSV disease?" Rather, the selection of a model will depend upon the type of RSV disease being studied. Problems of cost, availability, and opposition from animal rights activists will likely continue to restrict the study of RSV in primate models, particularly the chimpanzee. The primary advantage of the models are as follows: the calf, an RSV species in its natural host; the cotton rat, high permissiveness and reliability as a predictor of prophylactic and therapeutic strategies; the mouse, availability of specialized strains and reagents; the guinea pig, parallels in reactive airway disease; and the ferret, age-dependent pulmonary infection. Utilization of the calf, cotton rat, mouse, guinea pig, and ferret models likely will increase as the relative strengths of each model become better defined.

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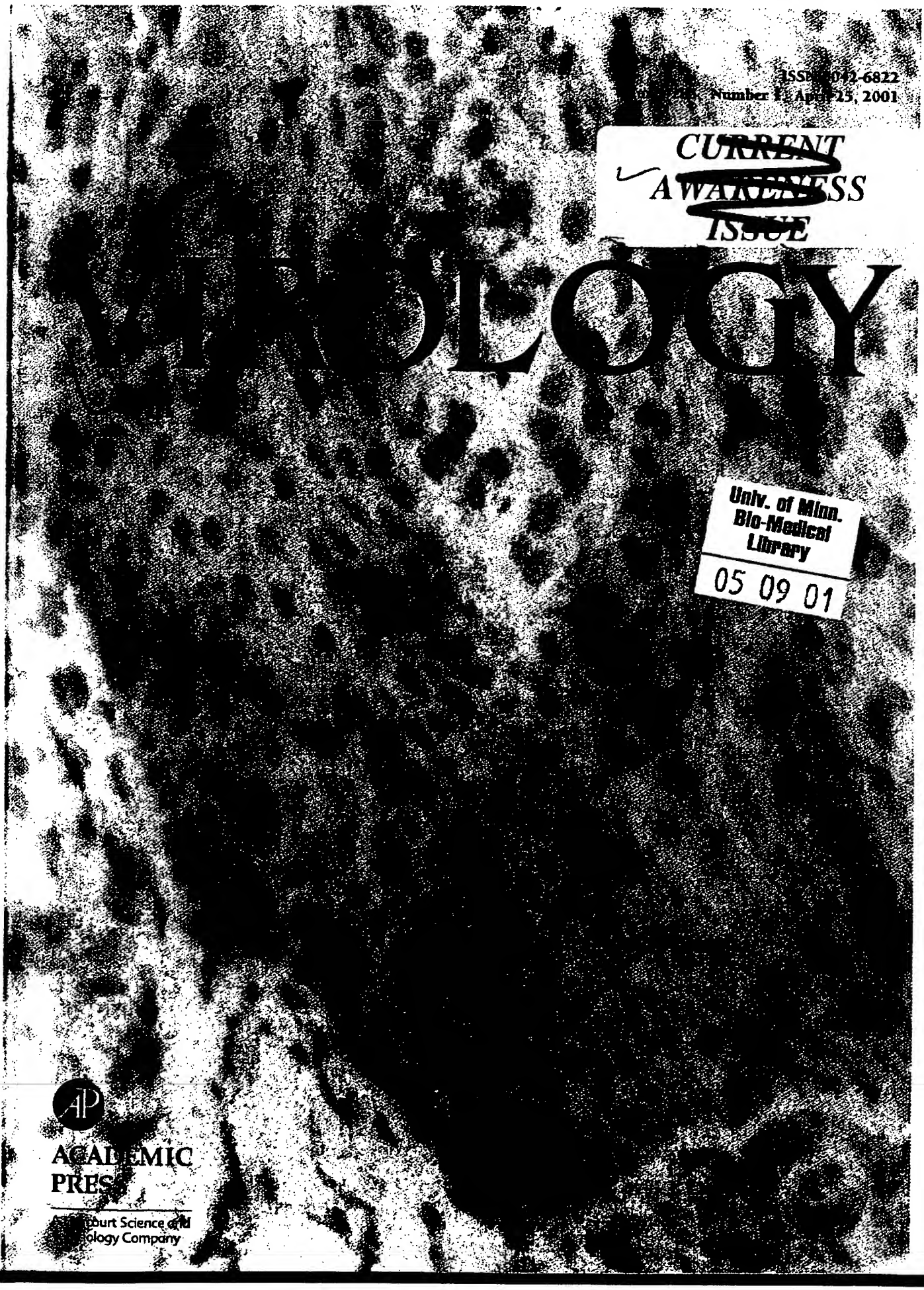

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Chimeric Subgroup A Respiratory Syncytial Virus with the Glycoproteins Substituted by Those of Subgroup B and RSV without the M2-2 Gene Are Attenuated in African Green Monkeys

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Using the existing reverse genetics system developed for the subgroup A respiratory syncytial virus (RSV), a chimeric virus (designated rA-G_BF_B) that expresses subgroup B-specific antigens was constructed by replacing the G and F genes of the A2 strain with those of the 9320 strain of subgroup B RSV. rA-G_BF_B grew well in tissue culture, but it was attenuated in the respiratory tracts of cotton rats and African green monkeys. To further attenuate this chimeric RSV, the M2-2 open reading frame was removed from rA-G_BF_B. rA-G_BF_BΔM2-2 was highly attenuated in replication in the respiratory tracts of the infected monkeys, but it provided complete protection against wild-type subgroup B RSV challenge following two doses of infection. In this study, rA2ΔM2-2 (a recombinant A2 RSV that lacks the M2-2 gene) was also evaluated in African green monkeys. The replication of rA2ΔM2-2 was highly restricted in both the upper and lower respiratory tracts of the infected monkeys and it induced titers of serum anti-RSV neutralizing antibody that were slightly lower than those induced by wild-type rA2. When rA2ΔM2-2-infected monkeys were challenged with wild-type A2 virus, the replication of the challenge virus was reduced by approximately 100-fold in the upper respiratory tract and 45,000-fold in the lower respiratory tracts. rA2ΔM2-2 and rA-G_BF_BΔM2-2 could represent a bivalent RSV vaccine composition for protection against multiple strains from the two RSV subgroups. © 2001 Academic Press

Key Words: recombinant respiratory syncytial virus; RSV vaccines; chimeric RSV; M2-2 deletion mutants; cotton rats; African green monkeys.

INTRODUCTION

Respiratory syncytial virus (RSV) is the leading cause of serious viral respiratory infection in infants and children worldwide. Despite decades of investigation, no safe and effective vaccines are available to prevent diseases caused by RSV infection. A number of live attenuated RSV candidate vaccines, generated by cold passage and/or chemical mutagenesis, have been evaluated in animals and humans (Crowe *et al.*, 1996a; Friedewald *et al.*, 1968; Gharpure *et al.*, 1969; Hsu *et al.*, 1995; Kim *et al.*, 1971; Richardson *et al.*, 1977). These previous vaccine candidates have been inadequately attenuated and in some circumstances genetically unstable, rendering them unsafe for young children (Hodes *et al.*, 1974; Kim *et al.*, 1973; Wright *et al.*, 1976, 2000). Recently, using the reverse genetics system developed for RSV (Collins *et al.*, 1995; Jin *et al.*, 1998), a large panel of cDNA-derived attenuated RSV have been obtained (reviewed by Collins *et al.*, 1999). A number of these attenuated RSV strains are currently being evaluated for use as vaccines.

RSV is an enveloped virus and contains a single-stranded, negative-sense RNA genome of 15,222 nucleotides (nt). Ten subgenomic mRNAs are encoded by the viral genome and are translated into 11 proteins: the nucleoprotein (N), the phosphoprotein (P), the major polymerase protein (L), the matrix protein (M), the glycoprotein (G), the fusion protein (F), two nonstructural proteins (NS1 and NS2), the small hydrophobic protein (SH), and the M2-1 and M2-2 proteins.

The G and F proteins are the major RSV surface antigens that elicit neutralizing antibodies *in vivo*. Two antigenically diverse RSV subgroups (A and B) have been distinguished on the basis of antigenic and sequence divergence. Within either subgroup, the G and F proteins exhibit high degrees of antigenic similarity. However, between subgroups, extensive differences are observed for the G protein. The antigenic diversity for the G protein between the two subgroups can be as great as 95% (Johnson *et al.*, 1987b). The F glycoprotein is relatively more conserved between the two subgroups. Although there is 91% identity between the amino acid sequences of the subgroup A and B F protein, the antigenic diversity can differ by as much as 50% (Johnson *et al.*, 1987a). This antigenic diversity enables both subgroups to circulate in a community at the same time (Reviewed by McIntosh and Chanock, 1990; Sullender, 2000). Although infection with subgroup A or B RSV in experimental animals induces a high level of resistance against replication of homologous or heterologous sub-

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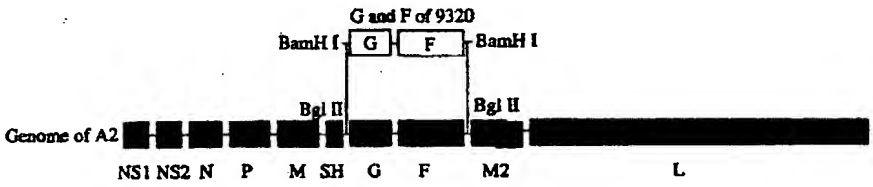

FIG. 1. Insertion of the G and F genes of RSV 9320 strain into recombinant A2 strain. The G and F genes of RSV 9320 were amplified by RT/PCR using primers that contained the introduced *Bam*HI restriction enzyme sites. The DNA cassette containing the G and F genes of 9320 was cloned into the *Bgl*II restriction sites that were created in the SH-G and F-M2 intergenic regions of the A2 strain.

group RSV, infection with attenuated RSV induced better protection against homotypic virus than heterotypic RSV (Crowe *et al.*, 1997a). Recent studies of a RSV vaccine in young infants have shown that infants develop dominant immune response against the RSV G protein than the F protein (Wright *et al.*, 2000). All these available data suggest that prevention of serious RSV diseases through vaccination would require bivalent vaccines containing antigenic components from both subgroups.

Previously, we described a reverse genetics system to generate recombinant RSV from cDNA (Jin *et al.*, 1998) and have used this technology to attenuate subgroup A RSV (Jin *et al.*, 2000a,b). To expedite the development of an attenuated subgroup B RSV vaccine, we used the infectious cDNA developed for the A2 strain to express heterologous subgroup B specific antigens. The construction of a chimeric RSV that expressed an additional G protein from a subgroup B virus in a recombinant A2 virus was described earlier (Jin *et al.*, 1998). Here we describe a recombinant chimeric RSV, designated rA-G_BF_B, in which the G and F genes of subgroup A were replaced with those of subgroup B. This virus is designated rA-G_BF_B. In addition, the M2-2 gene was removed from rA-G_BF_B, designated rA-$G_BF_B\Delta$M2-2, and this virus was shown to be more attenuated than rA-G_BF_B.

African green monkeys (AGM) were evaluated as a nonhuman primate model for assessing the attenuation, immunogenicity, and protective efficacy of RSV vaccine candidates. We showed that rA2 replicated to high titers in both the upper and the lower respiratory tracts of AGM, whereas rA2ΔM2-2, rA-G_BF_B, and rA-$G_BF_B\Delta$M2-2 replicated poorly in the respiratory tracts of monkeys. However, they all induced sufficient immunity to protect animals from experimental challenge.

RESULTS

Construction of cDNA and recovery of RSV A/B chimeric virus

Previously, we constructed an infectious antigenomic cDNA encoding wt RSV strain A2 and its derivative bearing a deletion of the M2-2 gene. Here, these cDNAs were modified by replacing the G and F genes of the A2 strain with those of the subgroup B RSV 9320 strain to produce chimeric viruses expressing RSV subgroup B antigens. The gene-start and gene-end sequences are very conserved between the two RSV subgroups. Therefore, the complete G and F genes of 9320 including their own gene-start and gene-end signals were transferred to the A2 cDNA backbone (Fig. 1). The cDNA encoding the G and F genes of 9320 was obtained by RT/PCR and confirmed by sequence analysis. The constructed chimeric cDNA was designated pRSVA-G_BF_B. pRSVA-$G_BF_B\Delta$M2-2 was constructed by deleting the M2-2 gene from pRSVA-G_BF_B. The M2 gene containing the deletion of the M2-2 open reading frame from rA2ΔM2-2 (Jin *et al.*, 2000a) was introduced into pRSVA-G_BF_B through the unique *Msc*I and *Bam*HI restriction enzyme sites. Both chimeric viruses (rA-G_BF_B and rA-$G_BF_B\Delta$M2-2) were recovered from cDNA using the previously described rescue system (Jin *et al.*, 1998). The recovered recombinant viruses were plaque-purified and amplified in Vero cells.

Characterization of the recombinant chimeric viruses *in vitro*

Expression of the subgroup specific proteins by the chimeric viruses was analyzed by Northern and Western blotting. Using strain-specific probes, 9320-specific G and F mRNAs were detected in cells infected with rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 (Fig. 2A). The M2-2 gene was not detected in cells infected with rA-$G_BF_B\Delta$M2-2 (lane 5), confirming that the M2-2 gene was deleted from this chimeric virus. The 9320 strain-specific protein expression of the two chimeric viruses was also compared with that of rA2, rA2ΔM2-2, and wild-type 9320 (Fig. 2B). The F1 protein of rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 showed the same rate of migration mobility as that of 9320; both migrated faster than that of A2. Western blotting analysis using strain-specific monoclonal antibodies confirmed that the G protein of subgroup B was expressed by rA-G_BF_B and rA-$G_BF_B\Delta$M2-2 (Fig. 2B). Immunoprecipitation using a polyclonal antibody specific to the M2-2 protein further confirmed the ablation of the M2-2 gene in rA2ΔM2-2 and rA-$G_BF_B\Delta$M2-2. The M2-2 protein of RSV strain 9320 was not detected by the polyclonal antiserum raised against the M2-2 protein of strain A2 as there is only 62% homology between the M2-2 proteins of the two RSV subgroups.

Replication of chimeric viruses, rA-G_BF_B and rA-

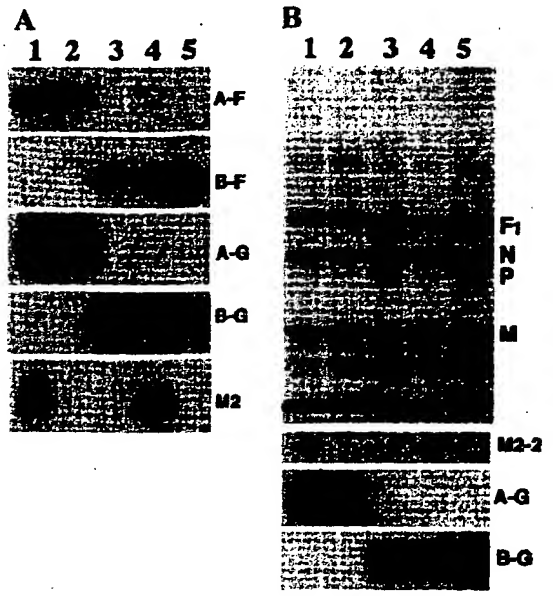

FIG. 2. Strain-specific expression of the chimeric RSV rA-G_BF_B and rA-G_BF_BΔM2-2. (A) Viral RNA expression. Total cellular RNA were extracted from virus-infected Vero cells and the Northern blots were hybridized with probes specific to the G or F gene of either subgroup A or subgroup B RSV. The M2-2 gene expression was examined by using a riboprobe specific to the M2-2 open reading frame. (B) Viral protein expression. The infected Vero cells were labeled with ^{35}S-methionine and ^{35}S-cysteine and the cell lysate immunoprecipitated with anti-RSV polyclonal antibody or anti-M2-2 antibody. To detect the G protein expression, the infected cell extracts were subjected to Western blotting using subgroup-specific monoclonal antibody against the G protein. Both rA-G_BF_B and rA-G_BF_BΔM2-2 expressed the subgroup B-specific G and F proteins and retained normal expression of the other genes derived from the subgroup A2 backbone. No M2-2 protein was expressed in rA2ΔM2-2 and rA-G_BF_BΔM2-2 infected cells. The M2-2 protein of strain 9320 was not recognized by the polyclonal serum raised against the M2-2 protein of strain A2. Lane 1: rA2; lane 2: rA2ΔM2-2; lane 3: 9320; lane 4: rA-G_BF_B; lane 5: rA-G_BF_BΔM2-2.

G_BF_BΔM2-2, was compared to rA2 and rA2ΔM2-2 in both the HEp-2 and the Vero cells (Fig. 3). In Vero cells, infected at an m.o.i. of 0.1, both rA-G_BF_B and rA-G_BF_BΔM2-2 reached peak titers similar to that seen with wild-type rA2 and rA2ΔM2-2, respectively. At a lower m.o.i. of 0.01, the peak titer of rA-G_BF_B was slightly reduced compared to rA2; the level of replication of rA-G_BF_BΔM2-2 was reduced by about 10-fold compared to rA-G_BF_B. In HEp-2 cells, at m.o.i. of 0.1, rA-G_BF_B showed a slightly lower peak titer compared to wt A2, whereas the replication of rA-G_BF_BΔM2-2 was reduced by about 100-fold. At m.o.i. of 0.01, the peak titer of rA-G_BF_B was reduced by about 10-fold compared to rA2 and the peak titer of rA-G_BF_BΔM2-2 was reduced by 100-fold. Therefore, similar to that observed for rA2ΔM2-2, rA-G_BF_BΔM2-2 also exhibited restricted replication in HEp-2 cells, whereas its replication in Vero cells was less impaired.

Replication of chimeric RSV in cotton rats

Cotton rats are susceptible to both subgroup A and subgroup B RSV infection. The levels of replication of rA-G_BF_B and rA-G_BF_BΔM2-2 in the nasal turbinates and lungs of cotton rats were compared with rA2, rA2ΔM2-2, and wild-type 9320 (Table 1). The replication of rA-G_BF_B was below the limit of detection by plaque assay in the nasal turbinates; its replication in lung tissue was reduced by about 3.6 $\log_{10}$ compared to wild-type 9320 and by about 2.0 $\log_{10}$ relative to rA2. The replication of rA2ΔM2-2 was not detected in the nasal turbinates and was 1.6 log lower in the lung compared to rA2. Removal of M2-2 from rA-G_BF_B further attenuated the chimeric virus. No virus replication was detected in either the nasal turbinates or the lungs of cotton rats infected with rA-G_BF_BΔM2-2.

Although rA-G_BF_B and rA-G_BF_BΔM2-2 were attenuated in cotton rats, both chimeric viruses induced sufficient immunity to protect the animals from homologous and heterologous RSV challenge (Table 1). rA-G_BF_BΔM2-2 induced complete protection against subgroup B RSV challenge, but its protection against the heterotypic subgroup A RSV challenge was incomplete in cotton rats. A low level of A2 challenge virus replication was detected in the nasal turbinates of cotton rats previously infected with rA-G_BF_BΔM2-2. The level of serum anti-RSV neutralizing antibody induced by rA-G_BF_B was 2.85-fold lower relative to that induced by wild-type 9320. Serum anti-RSV neutralizing antibody induced by rA-G_BF_BΔM2-2 was approximately fourfold lower compared to that induced by 9320 and 1.5-fold lower than that of rA-G_BF_B. By comparison, the level of serum anti-RSV neutralizing antibody induced by rA2ΔM2-2 was similarly reduced by approximately twofold compared to that of rA2.

Replication of wt RSV and rA2ΔM2-2 in AGM

To investigate RSV attenuation and immunogenicity in primates, replication of recombinant RSV was further studied in AGM. Study A examined the replication of recombinant A2 and wild-type A2 virus in the respiratory tracts of AGM. RSV seronegative AGM were infected with 5.5 $\log_{10}$ pfu of rA2 or wt A2 intranasally and intratracheally and virus shedding was monitored over a period of 12 days in both the upper and the lower respiratory tracts. As shown in Table 2, rA2 replicated well in both the upper and the lower respiratory tracts of AGM. rA2 reached a peak titer of 4.18 and 4.28 $\log_{10}$ pfu/ml at each site, respectively, and shed virus over the same length of time as the wild-type A2 virus (Table 2, study A), though the peak titer of rA2 in the respiratory tracts of AGM was slightly lower than that obtained for wild-type A2 virus. Having confirmed a high level of replication of rA2 in AGM, rA2ΔM2-2 was evaluated for its attenuation, immunogenicity, and protective efficacy in AGM. In a separate study (study B, Table 2), rA2ΔM2-2 showed a

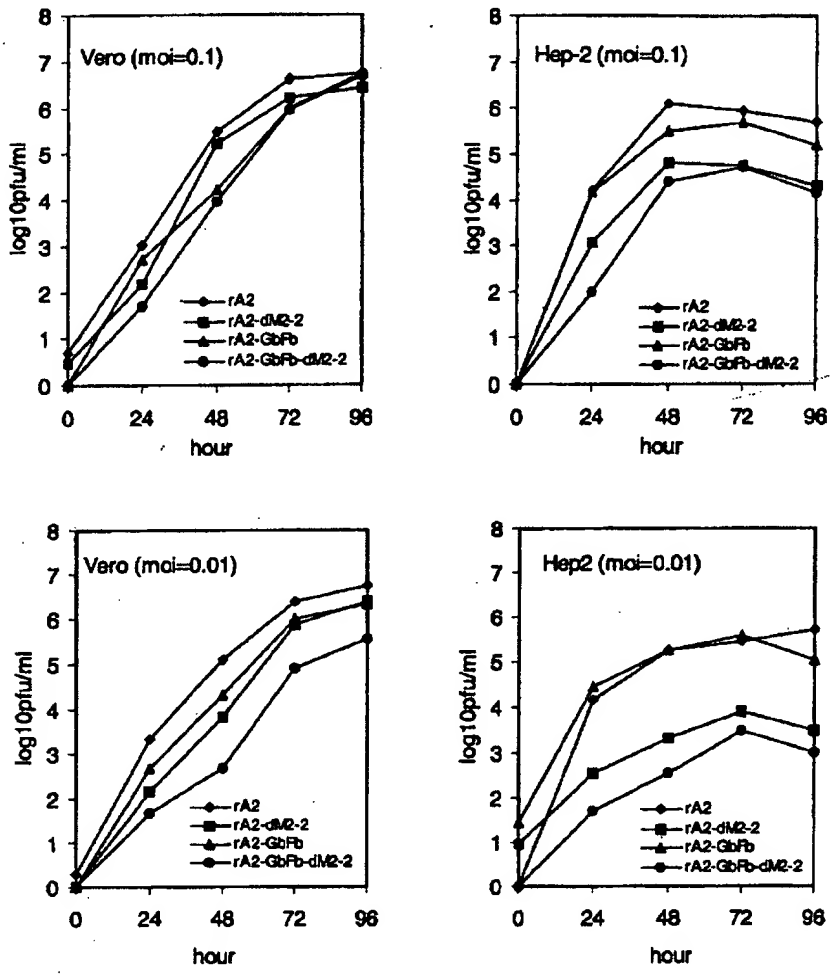

FIG. 3. Growth kinetics of the chimeric viruses in Hep-2 and Vero cells. Hep-2 or Vero cells were infected with viruses in duplicates at an m.o.i. of either 0.1 or 0.01. At 24 h intervals, the infected culture supernatants were harvested and virus titers determined by plaque assay in Vero cells.

greatly reduced level of replication in both the nasopharynx and the trachea compared to rA2. Its peak titer in nasopharynx was reduced by 3.1 $\log_{10}$, whereas the peak titer in the trachea was reduced by 3.25 $\log_{10}$ compared to rA2. Despite the much lower level of replication in the respiratory tracts, rA2ΔM2-2 induced a significant level of serum anti-RSV neutralizing antibody. The antibody titer induced by rA2ΔM2-2 was about fourfold lower than that induced by rA2 at 3 weeks postinfection (Table 3). When challenged with wild-type A2 virus, rA2ΔM2-2 provided partial protection against wild-type RSV replication in the upper respiratory tract and much greater protection in the lower respiratory tract of immunized monkeys. Monkeys previously infected with rA2 were fully protected against wt A2 virus replication in both the upper and the lower respiratory tracts (Table 3). Although rA2ΔM2-2 did not provide complete protection in the respiratory tracts of immunized monkeys, it reduced virus shedding by 5 days. Two weeks after challenge, the level of serum anti-RSV neutralizing antibody from rA2ΔM2-2 infected monkeys approached that induced by rA2.

Replication of chimeric RSV and wild-type 9320 in AGM

We next compared the level of replication of chimeric rA-G_BF_B with that of wild-type 9320. RSV seronegative AGM were inoculated with 5.5 $\log_{10}$ pfu of rA-G_BF_B or 9320 by intranasal and intratracheal instillation. The throat swab and tracheal lavage samples were collected over 12 days for virus quantitation. 9320 replicated to a level similar to that of wild-type A2 virus (Table 2). The peak titer of rA-G_BF_B at both sites of the respiratory tracts of the infected monkeys was about 1000-fold reduced compared to that of 9320. Animals infected with rA-G_BF_B shed virus for a shorter period of time than those infected with 9320. Despite its significantly attenuated replication, rA-G_BF_B provided complete protection when challenged with wild-type 9320. No challenge virus was detected in

TABLE 1

Replication, Immunogenicity, and Protection of Recombinant RSV against wt RSV Infection in the Upper and Lower Respiratory Tracts of Cotton Rats

Virus[a]	Virus titer[b] (mean $\log_{10}$pfu/g ± SE)		Neutralizing Ab titer (mean reciprocal $\log_2$)[c]	Titer of challenge virus (mean $\log_{10}$pfu/g ± SE)[d]			
				A2		9320	
	NT	Lung		NT	Lung	NT	Lung
rA2	3.9 ± 0.13	3.57 ± 0.07	10.0	<1.4	<1.4	ND[e]	ND
rA2ΔM2-2	<1.4	2.02 ± 0.12	9.0	<1.4	<1.4	ND	ND
Control	<1.4	<1.4	<3.3	4.2 ± 0.14	6.0 ± 0.06	2.3 ± 0.53	5.2 ± 0.01
9320	2.8 ± 0.57	5.8 ± 0.05	10.64	<1.4	<1.4	<1.4	<1.4
rA-G_9F_9	<1.4	1.94 ± 0.31	9.13	<1.4	<1.4	<1.4	<1.4
rA-G_9F_9ΔM2-2	<1.4	<1.4	8.57	1.2 ± 0.65	<1.4	<1.4	<1.4

[a] Cotton rats were administered with 5.5 $\log_{10}$PFU of virus intranasally under light anesthesia on day 0 and sacrificed on day 4.

[b] Virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.

[c] Serum RSV neutralizing antibody titers were determined by a complement-enhanced 50% plaque reduction assay with wt A2 or 9320.

[d] On day 21 of virus infection, cotton rats in groups of six were challenged with wt A2 or wt 9320 and the challenge virus titers from the nasal turbinates (NT) and lung tissues were determined by plaque assay.

[e] ND, not determined.

either the upper or the lower respiratory tracts of the monkeys previously immunized with rA-G_9F_9 (Table 3). Consistent with the level of protection seen in monkeys immunized with rA-G_9F_9, the level of serum anti-RSV neutralizing antibody from these monkeys was similar to that observed for wild-type 9320-infected animals.

rA-G_9F_9ΔM2-2 was evaluated in AGM in a separate study (study C). The replication of rA-G_9F_9ΔM2-2 was not detected in the upper respiratory tracts and a very low level of virus replication was detected in the lower respiratory tracts of the infected monkeys (Table 2). Since rA-G_9F_9ΔM2-2 appeared to be more attenuated than rA-G_9F_9 and rA2-ΔM2-2, an additional boosting dose was administered 4 weeks later. The boosting infection greatly augmented immune response and provided complete protection against wild-type 9320 RSV challenge. The level of serum anti-RSV neutralizing antibody induced by rA-G_9F_9ΔM2-2 was about fourfold lower than that induced by rA-G_9F_9. However, after a second dose of boosting infection, the level of serum neutralizing antibody was increased by about eightfold and it was further augmented by an additional twofold following subsequent wild-type RSV infections.

DISCUSSION

In an attempt to develop live attenuated RSV vaccine, we are using a recently developed reverse genetics system to attenuate RSV by introducing various mutations into the RSV genome. This approach has generated a number of attenuated subgroup A recombinant RSV by different groups (Jin *et al.*, 2000a,b; Teng and Collins, 1999; Teng *et al.*, 2000; Bermingham and Collins, 1999; Whitehead *et al.*, 1999a,b). To expedite vaccine develop-

TABLE 2

Replication of Recombinant RSV in the Upper and Lower Respiratory Tracts of African Green Monkeys

Virus[a]	AGM number	Virus shedding (days)	Virus peak titer (Mean $\log_{10}$pfu ± SE)[b]	
			Nasopharyngeal swab	Tracheal lavage
wt A2	4 (Study A)	8	4.67 ± 0.17	4.97 ± 0.04
rA2	4 (Study A)	8	4.18 ± 0.18	4.28 ± 0.27
rA2	4 (Study B)	9	3.44 ± 0.27	3.91 ± 0.18
rA2ΔM2-2	4 (Study B)	4	0.33 ± 0.26	0.66 ± 0.40
9320	4 (Study B)	9	4.51 ± 0.18	4.36 ± 0.45
rA-G_9F_9	4 (Study B)	4	1.50 ± 0.42	1.77 ± 0.25
rA-G_9F_9ΔM2-2	4 (Study C)	3	<0.7	0.25 ± 0.25

[a] African green monkeys were administered with 5.5 $\log_{10}$PFU of virus intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal-lavage samples were collected on days 3, 5, 7, and 10.

[b] Virus titers were determined in the nasopharyngeal swab and tracheal-lavage by plaque assay and only the peak titers are shown.

TABLE 3

Evaluation of Recombinant RSV for Their Levels of Immunogenicity and Efficacy against Wild Type Challenge Virus in African Green Monkeys

Virus	Challenge virus[a]	Virus peak titer (Mean $\log_{10}$pfu ± SE)[b]		Neutralizing Ab titer (Mean reciprocal $\log_2$)[c]		
		Nasopharyngeal swab	Tracheal lavage	Day 0	Day 28	Day 42
rA2	A2	<0.7	<0.7	<3.3	9.7	10.5
rA2ΔM2-2	A2	2.64 ± 0.07	0.46 ± 0.47	<3.3	7.7	9.25
None	A2	4.67 ± 0.17	4.97 ± 0.04	<3.3	<3.3	10.5
9320	9320	<0.7	<0.7	<3.3	7.0	10.0
rA-G_BF_B	9320	<0.7	<0.7	<3.3	7.75	10.5
rA-G_BF_BΔM2-2[d]	9320	<0.7	<0.7	<3.3	5.5	8.75[d]
None	9320	4.51 ± 0.18	4.36 ± 0.35	<3.3	<3.3	10.0

[a] African green monkeys were administered with 5.5 $\log_{10}$PFU of virus intranasally and intratracheally and on day 28; monkeys were challenged with wt A2 or wt 9320 at a dose of 5.5 $\log_{10}$PFU intranasally and intratracheally.

[b] Nasopharyngeal swab samples were collected daily for 10 days, and tracheal-lavage samples were collected on days 3, 5, 7, and 10. Challenge virus titers were determined by plaque assay. Only the peak titers are shown.

[c] Serum RSV neutralizing antibody titers from monkeys infected with rA2 and rA2ΔM2-2 before challenge infection (day 28) and 14 days post challenge (day 42) were determined by a complement-enhanced 50% plaque reduction assay with wt A2. The neutralizing antibody titers from monkeys infected with 9320, rA-G_BF_B, and rA-G_BF_BΔM2-2 were determined by microneutralization assay (Cheng *et al.*, manuscript in preparation).

[d] An additional boosting dose was administered; shown here is postboosting neutralizing antibody titer at day 56. The antibody titer after challenge (day 70) was 9.75 $\log_2$.

ment for subgroup B RSV, we used recombinant A2 virus as a vector to express subgroup B RSV surface antigens. The chimeric virus should elicit a balanced immune response and provide protection against subgroup B RSV infection. Previously, we described a chimeric virus, A2(B-G), that expressed two G proteins, one derived from A2 strain and the other derived from 9320 strain (Jin *et al.*, 1998). A2(B-G) provided complete protection against subsequent wild-type RSV A and B strains infection in cotton rats. While this chimeric virus retained both G genes of two subgroups during passage in tissue culture, frameshift mutations have been detected at oligo(A) tracts of one or the other subgroup G gene on separate occasions during *in vitro* passages. As an alternative approach to expressing RSV subgroup B antigens, we constructed a different chimeric virus in which the G and F genes of the A2 strain were completely replaced by the G and F genes of the 9320 strain. The chimeric RSV was then further attenuated using a strategy developed for attenuating the A2 virus.

The recovered chimeric RSV (rA-G_BF_B) replicated efficiently in Vero cells, but its growth in HEp-2 cells was reduced by 5- to 10-fold relative to rA2. rA-G_BF_B was attenuated in both the upper and the lower respiratory tracts of cotton rats. To determine whether the attenuation of rA-G_BF_B was host specific, this chimeric virus was further evaluated in AGM that are genetically more closely related to humans than rodents. RSV infection in AGM is less well characterized and there is a wide range in the reported peak titer (Crowe *et al.*, 1996b; Kakuk *et al.*, 1993). Therefore, we first tested RSV infection in AGM using wild-type viruses. We showed that both subgroup A and subgroup B RSV replicated equally well in AGM and virus titers recovered from the upper and lower respiratory tracts of AGM approached those observed in infected chimpanzees (Crowe *et al.*, 1994). When rA-G_BF_B was evaluated in AGM, it showed a mean peak titer reduction of 3.0 $\log_{10}$ in the upper respiratory tract and a reduction of 2.59 $\log_{10}$ in the lower respiratory tract.

The level of attenuation of rA-G_BF_B in AGM was consistent with what we observed in cotton rats. However, this result was somewhat different from that reported for a recently described chimeric RSV in which the G and F genes of A2 were replaced with those of RSV B1 strain (rAB1) (Whitehead *et al.*, 1999b). Though rAB1 and rA-G_BF_B are similarly attenuated in cotton rats, rAB1 was not attenuated in chimpanzees. In contrast to rA-G_BF_B, rAB1 replicated better than wt RSV B1 in both the upper and the lower respiratory tracts of chimpanzees (Whitehead *et al.*, 1999b). Part of this discrepancy may be explained by the semipermissiveness of chimpanzees to wild-type subgroup B RSV infection. However, there exists the possibility that rA-G_BF_B is more attenuated than rAB1 because of differences in the subgroup B strain surface antigens or constellation effects when these antigens are introduced into an A2 background. Chimerization of surface antigens resulting in an attenuated virus has been reported for several paramyxoviruses. A chimeric measles virus with the HN and F proteins replaced by the G protein of VSV was highly restricted in replication *in vitro* (Spielhofer *et al.*, 1998). A chimeric Rinderpest virus in which the F and H proteins were replaced by the heterologous surface proteins of a closely related pestedes-petits-ruminants virus was attenuated *in vitro*, as indicated by slow virus growth and low virus yield (Das *et al.*, 2000). Most recently, it was reported that the PIV3-

PIV2 chimeric virus, in which the F and HN genes of PIV3 were replaced by those of PIV2, was not attenuated *in vitro*, but it was highly attenuated in hamsters, AGM, and chimpanzees (Tao *et al.*, 2000). On the other hand, the chimeric PIV3-PIV1 was not attenuated *in vivo* (Tao *et al.*, 1998, 1999). Thus, it appears that chimerization of different heterologous proteins can result in different phenotypes. Though attenuated in AGM, rA-G_BF_B induced significant levels of anti-RSV neutralizing antibody and provided complete protection against subsequent challenge with wild-type subgroup B RSV.

We previously reported that the recombinant A2 RSV lacking the M2-2 gene is attenuated in mice and cotton rats. In this study, we evaluated rA2ΔM2-2 for its attenuation, immunogenicity, and protection against wild-type RSV challenge in AGM. We showed that rA2ΔM2-2 was attenuated in the respiratory tracts of AGM and following challenge, much reduced replication of wild-type RSV was observed in animals previously infected with rA2ΔM2-2. The protection was higher in the lower respiratory tract than the upper respiratory tract. The level of replication and protection observed for rA2ΔM2-2 in AGM is very similar to that reported in a chimpanzee study for a similar recombinant RSV that had the M2-2 protein expression silenced (Bermingham and Collins, 1999; Teng *et al.*, 2000). rA2ΔM2-2 may prove to be more attenuated in humans than a previously tested vaccine candidate cpts248/404 (Teng *et al.*, 2000). cpts248/404 was neither sufficiently attenuated nor genetically stable in naive infants (Crowe *et al.*, 1994; Wright *et al.*, 2000). The serum anti-RSV neutralizing antibody titer induced by rA2ΔM2-2 was slightly lower than that induced by the wild-type RSV infection. However, the augmentation of neutralizing antibody titer after the challenge suggests that the immunogenicity of rA2ΔM2-2 could be enhanced by repeat administrations.

Since rA2ΔM2-2 exhibits many of the desired features in a live attenuated vaccine, we considered the deletion of the M2-2 gene an appropriate way to further attenuate the chimeric rA-G_BF_B. *In vitro* studies indicated that rA-G_BF_BΔM2-2 had phenotypes similar to rA2ΔM2-2, exhibiting increased syncytial formation, reduced growth in HEp-2 cells, and unbalanced RNA transcription to replication. rA-G_BF_BΔM2-2 was more attenuated than rA-G_BF_B and rA2ΔM2-2 in both cotton rats and AGM. This attenuated replication in the host led to its reduced immunogenicity. Thus, although rA-G_BF_B provided complete protection to both subgroup A and subgroup B RSV challenge, A-G_BF_BΔM2-2 provided lower protection to subgroup A virus infection than to subgroup B strain infection in cotton rats. A very low level of serum anti-RSV neutralizing antibody was detected in monkeys infected with rA-G_BF_BΔM2-2. The second dose administration of A-G_BF_BΔM2-2 greatly augmented antibody response and provided complete protection against subsequent experimental challenge in AGM. Our data implied that a live attenuated RSV vaccine containing components from both subgroups need to be administered in multiple doses to achieve a higher level of durable immunity. rA-G_BF_BΔM2-2, in combination with rA2ΔM2-2, may represent suitable vaccines for protecting against both subgroup A and subgroup B RSV infections.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of HEp-2 and Vero cells (obtained from American Type Culture Collections, ATCC) were maintained in minimal essential medium (MEM) containing 5% fetal bovine serum (FBS). Wild-type RSV strains, A2 and 9320, were obtained from ATCC and grown in Vero cells. Modified vaccinia virus Ankara (MVA-T7) expressing bacteriophage T7 RNA polymerase was provided by Dr. Bernard Moss and grown in CEK cells.

Construction of chimeric cDNA clone

The wild-type RSV strain 9320, originally isolated in Massachusetts in 1977 and classified as subgroup B RSV (Hierholzer and Hirsch, 1979), was used in this study. The 9320 RSV was grown in Vero cells and the viral RNA was extracted from infected cell culture supernatant. A cDNA fragment containing the G and F genes of RSV 9320 was obtained by RT/PCR using the following primers: ATCA*GGATCCACAATAACATTGGGGCAAATGCAACC* and CTGGCATTC*GGATCCGTTTTATGTAACTATGAGTTG* (the *Bam*HI sites engineered for cloning are in italics and 9320 specific sequences are underlined). *Bam*HI restriction enzyme sites were introduced upstream of the gene start sequence of G and downstream of the gene end sequence of F. The PCR product was first introduced into the T/A cloning vector (Invitrogen) and the sequences were confirmed by DNA sequencing. The *Bam*HI restriction fragment containing the G and F gene cassette of 9320 was then transferred into a RSV cDNA subclone pRSV(R/H) that contained RSV sequences from nt 4326 to nt 9721 through the introduced *Bgl*II sites at nt 4655 (upstream of the gene start signal of G) and at nt 7552 (downstream of the gene end signal of F). Introduction of these two *Bgl*II sites were made by PCR mutagenesis using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA). *Bam*HI and *Bgl*II restriction enzyme sites have compatible ends but ligation obliterates both restriction sites. The *Xho*I (nt 4477) to *Bam*HI (nt 8498) restriction fragment containing the G and F genes of 9320 was examined by sequencing analysis and then shuttled into the infectious RSV antigenomic cDNA clone pRSVC4G (Jin *et al.*, 1998). The chimeric antigenomic cDNA was designated pRSV-G_BF_B. To delete the M2-2 gene from pRSV-G_BF_B, the Msc I (nt 7692) to *Bam*HI (nt 8498) fragment from rA2ΔM2-2 which contained the

M2-2 deletion (Jin *et al.*, 2000a) was introduced into pRSV-G_BF_B. The chimeric cDNA clone that lacks the M2-2 gene was designated pRSV-$G_BF_B\Delta$M2-2.

Recovery of recombinant RSV

Recovery of recombinant RSV from cDNA was described previously (Jin *et al.*, 1998). Briefly, HEp-2 cells in six-well plates at 80% confluence were infected with MVA at an m.o.i. of 5 pfu/cell for 1 h and then were transfected with full-length antigenomic plasmids (pRSV-G_BF_B or pRSV-$G_2F_B\Delta$M2-2), together with plasmids expressing the RSV N, P, and L proteins using LipofecTACE reagent (Life Technologies, Gaithersburg, MD). After incubating the transfected cells at 35°C for 3 days, the culture supernatants were passaged in Vero cells for 6 days to amplify rescued virus. The recovered recombinant viruses were biologically cloned by three successive plaque purifications and further amplified in Vero cells. Virus recovered from pRSV-G_BF_B-transfected cells was designated rA-G_BF_B and that from pRSV-$G_BF_B\Delta$M2-2 transfected cells was designated rA-$G_BF_B\Delta$M2-2. Virus titer was determined by plaque assay and plaques were enumerated by immunostaining using polyclonal anti-RSV A2 serum (Biogenesis, Sandown, NH).

Virus characterization

The expression of viral RNA for each recovered chimeric RSV was analyzed by Northern blotting. Total cellular RNA was extracted from virus-infected cells at 48 h postinfection. The RNA blot was hybridized with a γ-[^{32}P]ATP-labeled oligonucleotide probe specific for the F gene of 9320 (GAGGTGAGGTACAATGCATTAATAGCAAGATGGAGGAAGA) or a γ-[^{32}P]ATP-labeled probe specific for the F gene of A2 (CAGAAGCAAAACAAAATGTGACTGCAGTGAGGATTGTGGT). To detect the G mRNA of the chimeric viruses, RNA blots were hybridized with a 190-nt riboprobe specific to the G gene of 9320 or a 130-nt riboprobe specific to the G gene of A2. Both riboprobes were labeled with α-[^{32}P]-CTP. Hybridization was performed at 65°C in Express Hyb solution (Clontech, Palo Alto, CA) overnight. Membranes were washed at 65°C under stringent conditions and exposed to film.

Viral specific proteins from infected cells were analyzed by immunoprecipitation of the infected cell extracts or by Western blotting. To immunoprecipitate viral proteins, Vero cells were infected with virus at an m.o.i. of 1.0 and labeled with ^{35}S-promix (100 μCi/ml ^{35}S-Cys and ^{35}S-Met; Amersham, Arlington Heights, IL) from 14 to 18 h postinfection. The labeled cell monolayers were lysed with RIPA buffer and the polypeptides immunoprecipitated by polyclonal goat anti-RSV A2 serum (Biogenesis) or by a polyclonal antibody against the M2-2 protein (Jin *et al.*, 2000a). Immunoprecipitated polypeptides were electrophoresed on SDS–PAGE and detected by autoradiography. For Western blotting analysis, virus-infected Vero cells were lysed in protein lysis buffer and the proteins were separated on 12% SDS–PAGE. The proteins were transferred to a nylon membrane and immunoblotting was performed as described (Jin *et al.*, 1997), using a monoclonal antibody recognizing the G protein of strain 9320 or a monoclonal antibody against the G protein of A2 (Storch and Park, 1987).

Growth of chimeric RSV *in vitro* was compared with wild-type recombinant A2 (rA2) and rA2ΔM2-2. Growth-cycle analysis was performed in both HEp-2 and Vero cells. Cells grown in 6-cm dishes were infected with each virus at a m.o.i. of 0.01 or 0.1. After 1 h absorption at room temperature, the infected cell monolayers were washed 3 times with PBS and incubated at 35°C with 4 ml of Opti-MEM in an incubator containing 5% CO_2. At various times postinfection, 200 μl of the culture supernatant was collected and stored at −70°C for virus titration. Each aliquot removed was replaced with an equal amount of fresh medium. Virus titer was determined by plaque assay in Vero cells on 12-well plates using an overlay of 1% methylcellulose and 1× L15 medium containing 2% FBS.

Virus replication in cotton rats

Virus replication *in vivo* was determined in respiratory pathogen-free *Sigmodon Hispidus* cotton rats. Cotton rats in groups of 12 or 18 were inoculated intranasally under light methoxyflurane anesthesia with $10^{6.5}$ pfu of virus per animal in a 0.1-ml inoculum. On day 4 postinoculation, six animals were sacrificed by CO_2 asphyxiation and their nasal turbinates and lungs were harvested separately. Tissues were homogenized and virus titers determined by plaque assay in Vero cells. Three weeks later, the remaining six animals were anesthetized, their serum samples were collected, and a challenge inoculation of 10^6 pfu of biologically derived wild-type RSV strain A2 or 9320 administered intranasally. To investigate the cross-protection of the chimeric viruses to heterologous RSV, six additional animals infected with rA-G_BF_B or rA-$G_BF_B\Delta$M2-2 were also challenged with wt A2 RSV. Four days postchallenge, the animals were sacrificed and both nasal turbinates and lungs were harvested, homogenized, and virus titer determined by plaque assay. Serum neutralizing antibodies against RSV A2 or 9320 strain were determined by a 50% plaque reduction assay (Coates *et al.*, 1966).

Virus replication in AGM

Recombinant RSV was evaluated for their replication, immunogenicity, and protective efficacy in AGM (*Cercopithecus aethiops*). AGM, obtained from St. Kitts with an average age of 4.2 years and body weight ranging from 2.2 to 4.3 kg, were used in the first study (study A) to compare the replication of rA2 with wild-type A2. The

second study (study B) used AGM with ages ranging from 5.3 to 8.4 years and an average body weight of 4.15 kg. None of the monkeys had detectable serum neutralizing antibodies for RSV 9320 or A2 (titer < 1:10). Groups of four monkeys were inoculated with either wild-type A2, rA2, rA2ΔM2-2, wild-type 9320, or rA-G_BF_B by both intranasal and intratracheal route with a dose of $10^{5.5}$ pfu in a 1.0 ml inoculum at each site. Following inoculation, daily nasopharyngeal (NP) swabs were collected from each monkey for 12 days under Telazol anesthesia and bronchoalveolar lavage (BAL) were collected on days 3, 5, 7, and 10 postinfection (Kakuk *et al.*, 1993). On day 28 postinfection, serum samples were collected from each infected monkey and the monkeys were challenged with either wild-type A2 or 9320 at both the intranasal and the intratracheal sites with a dose of $10^{5.5}$ pfu in a 1.0-ml inoculum. Replication of the challenge virus in the upper and lower respiratory tracts of monkeys was examined by quantitation of virus shed in NP and tracheal lavage specimens. The NP samples were collected daily for 10 days and BAL samples were collected on days 3, 5, 7, and 10 postchallenge. Fourteen days after wild-type virus challenge, serum samples were collected for measurement of RSV neutralizing antibody. rA-G_BF_BΔM2-2 was evaluated in a separate study (study C). Four week after infection, a group of four monkeys were administered with an additional boosting dose of 5.5 $\log_{10}$ pfu of rA-G_BF_BΔM2-2 intranasally and intratracheally and monkeys were challenged with 5.5 $\log_{10}$ pfu of wt 9320 virus 4 weeks after boosting infection. NP and BAL samples were collected from rA-G_BF_BΔM2-2-infected monkeys the same as described for those infected with rA-G_BF_B. Serum samples were collected at day 0 (preserum), day 28 (preboosting), day 56 (postboosting), and day 70 (postchallenge). The levels of neutralizing antibody from monkeys infected with rA2 and rA2ΔM2-2 were determined by the 50% plaque reduction assay using wild-type A2 virus. The levels of neutralizing antibody from monkeys infected with 9320, rA-G_BF_B, and rA-G_BF_BΔM2-2 were determined by a microneutralization assay (Cheng *et al.*, manuscript in preparation). The virus shedding in the NP and BAL samples were quantitated by plaque assay using Vero cells.

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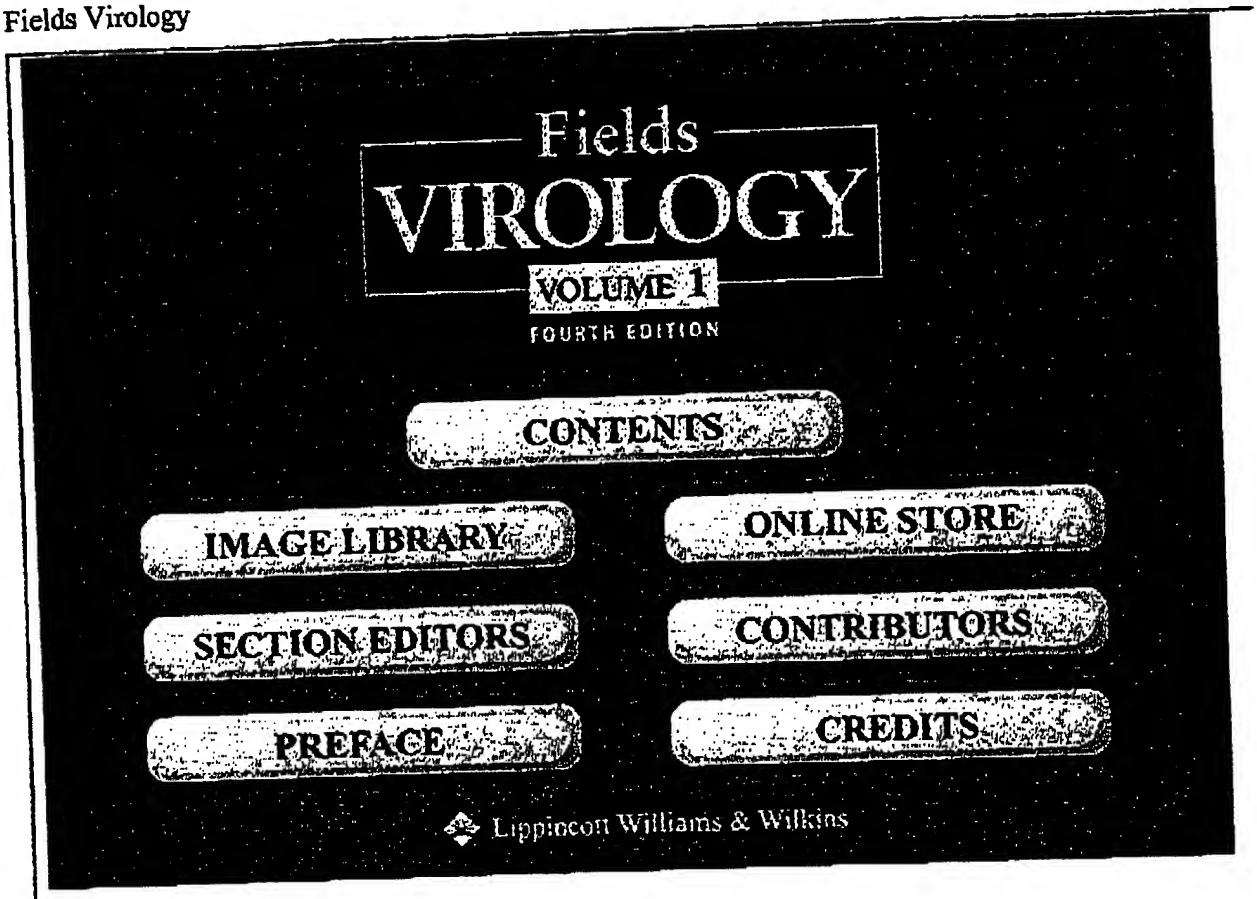

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Section Two: Specific Virus Families
Chapter 45: Respiratory Syncytial Virus
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Section Two: Specific Virus Families
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GENETICS AND REVERSE GENETICS

The frequency of isolation of MAb-resistant mutants (MARMs), selected with various MAbs specific to the F protein, was estimated to be 10^{-4} to 10^{-6}, which is similar to that observed with other RNA viruses (31). The actual mutation rate likely is higher, because lethal mutations would not have been recovered. Other information on the stability of RSV *in vitro* was obtained from studies designed to produce live-attenuated vaccines. In one study, 52 passages of strain A2 at progressively lower temperatures resulted in a cold-passaged (cp) RSV that had acquired only five point mutations (128,424). Detailed characterization of six temperature-sensitive (ts) attenuated mutants generated by chemical mutagenesis and biologic selection identified in each a single unique point mutation responsible for the ts and attenuation phenotype. One mutant also contained a single additional amino acid change that did not contribute to the ts or attenuation phenotype, and hence the introduction of incidental mutations into infectious virus was surprisingly rare (86,124,221,222,421,422).

The spontaneous deletion of the SH and G genes during *in vitro* passage, resulting in the B1 cp-52 virus, has already been mentioned (227). Interestingly, there was evidence for multiple independent deletion events in the virus population involving these two genes. Deletion of SH and G during this particular passage series presumably could be accommodated because neither gene is required for efficient growth in Vero cells, as mentioned previously. This propensity to delete G is puzzling because studies with recombinant RSV showed that inserts of foreign sequence are surprisingly stable, as has been found with other mononegaviruses (47,48). Point mutations accumulate in inserted foreign sequence at a low rate over multiple cycles of replication, but there was no evidence of deletion, and functional foreign protein was expressed by most recombinant RSV plaque isolates even after multiple passages.

Other types of unusual mutations also have been observed in RSV subjected to strong selective pressure *in vitro*. For example, multiple point mutations have been described in the NS2 or G genes that apparently were acquired by biased hypermutation (261,383). The finding that strain variation involves a disproportionately high frequency of A to G changes has been suggested to be evidence of hypermutation in nature (263). Frame shifting can occur at several A-rich sequences in the downstream half of the G gene, which accesses an alternative ORF. This has been detected by analysis of cloned cDNAs of viral RNA from infected cells (57), and also was identified in certain MARMs isolated *in vitro* (261) (see Antigenic Determinants). A single natural isolate had suggestive evidence of having sustained a frame shift (376), suggesting that this can occur in nature but is not common. The alternative ORF that is accessed by frame shifting is not thought to encode a functional domain. Other MARMs contained premature stop codons in the downstream end of the G ORF, and this might also occur in nature because certain natural isolates contain small C-terminal deletions (58,376). It is likely that these unusual types of mutations can be found in G because its structure is relatively tolerant of mutation.

Homologous recombination is not known to occur. Thus far, defective-interfering RSV genomes similar to those of other model mononegaviruses such as Sendai and vesicular stomatitis viruses have not been identified by molecular analysis, although there is indirect evidence for their existence (389).

Studies with RSV and mononegaviruses in general have been revolutionized by "reverse genetics," by which complete infectious recombinant virus can be recovered from cDNA. The production of recombinant RSV requires the intracellular coexpression, from transfected plasmids, of antigenomic RNA and the N, P, L, and M2-1 support proteins (44,77,79,208). The requirement for M2-1 as a fourth support protein distinguishes RSV from the paramyxoviruses (77). This defined for the first time a

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correct sequence for wt RSV, and provided the means to introduce predetermined changes into infectious virus via the cDNA intermediate.

A second type of reverse genetic system involves minireplicons, which are short cDNA-encoded versions of genomic or antigenomic RNA in which some or all of the viral genes have been deleted and replaced with one or more genes encoding marker proteins such as bacterial chloramphenicol acetyl transferase or luciferase. Minireplicons usually do not contain the genes necessary for their own replication and must be complemented by viral proteins provided by cotransfected plasmids or by coinfecting standard RSV. They are useful because it is easier to perform extensive mutational and biochemical analyses with a short replicon containing only one or a few genes instead of the complete genome. The fact that the minireplicon and support proteins are encoded by separate plasmids also means that each component can be manipulated separately without affecting the synthesis of the others.

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Recombinant Respiratory Syncytial Viruses with Deletions in the NS1, NS2, SH, and M2-2 Genes Are Attenuated *in Vitro* and *in Vivo*

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Respiratory syncytial virus (RSV) encodes several proteins that lack well-defined functions; these include NS1, NS2, SH, and M2-2. Previous work has demonstrated that NS2, SH, and M2-2 can each be deleted from RSV genome and thus are considered as accessory proteins. To determine whether RSV can replicate efficiently when two or more transcriptional units are deleted, we removed NS1, NS2, SH, and M2-2 genes individually and in different combinations from an infectious cDNA clone derived from human RSV A2 strain. The following six mutants with two or more genes deleted were obtained: ΔNS1NS2, ΔM2-2SH, ΔM2-2NS2, ΔSHNS1, ΔSHNS2, and ΔSHNS1NS2. Deletion of M2-2 together with NS1 was detrimental to RSV replication. It was not possible to obtain a recombinant RSV when all four genes were deleted. All of the double and triple deletion mutants exhibited reduced replication and small plaque morphology *in vitro*. Replication of these deletion mutants was more reduced in HEp-2 cells than in Vero cells. Among the 10 single and multiple gene deletion mutants obtained, ΔM2-2NS2 was most attenuated. ΔM2-2NS2 formed barely visible plaques in HEp-2 cells and had a reduction of titer of 3 $\log_{10}$ compared with the wild-type recombinant RSV in infected HEp-2 cells. When inoculated intranasally into cotton rats, all of the deletion mutants were attenuated in the respiratory tract. Our data indicated that the NS1, NS2, SH, and M2-2 proteins, although dispensable for virus replication *in vitro*, provide auxiliary functions for efficient RSV replication. © 2000 Academic Press

INTRODUCTION

Human respiratory syncytial virus (RSV) is the most important viral agent of serious respiratory tract disease in infants and children worldwide (McIntosh and Chanock, 1990). It is the prototype member of the *Pneumovirus* genus of the Paramyxoviridae family (Lamb and Kolakofsky, 1996). RSV is an enveloped nonsegmented negative-strand RNA virus. Virus replication involves synthesis of its positive-sense RNA intermediate (antigenome), which then serves as a template for amplification of the negative-sense virus genome. Although most members of the Mononegavirales possess only five or six transcription units, RSV contains 10 transcription units. Each transcription unit contains the 10-nucleotide gene start and 12- to 13-nucleotide gene end motifs for transcription. Ten mRNAs are sequentially transcribed from the RSV genome to produce 11 proteins in the following order: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L. The nucleoprotein (N), phosphoprotein (P), and the major polymerase protein (L) form the minimal viral polymerase complex for viral RNA transcription and replication (Yu *et al.*, 1995; Grosfeld *et al.*, 1995). The matrix protein (M) is a peripheral membrane protein located between viral nucleocapsids and viral envelope and is involved in virion morphogenesis. The attachment (G) and fusion (F) proteins are the two major viral surface glycoproteins that are involved in virus assembly, budding, and entry.

NS1, NS2, SH, M2-1, and M2-2 are the five additional proteins encoded by the pneumoviruses. Except for the SH (small hydrophobic) protein that has counterparts in the rubulaviruses SV5 (He *et al.*, 1998; Hiebert *et al.*, 1985) and mumps virus (Elango *et al.*, 1989), the NS1, NS2, M2-1, and M2-2 proteins lack obvious counterparts in other nonsegmented negative-strand RNA viruses. The transcripts of NS1 and NS2 are two of the most abundantly expressed RNAs in RSV-infected cells due to their promoter-proximal location. The NS1 gene of RSV strain A2 is 552 nt long and encodes a protein of 139 amino acids (Collins and Wertz, 1985). The NS2 gene is 503 nt long and encodes a protein of 124 amino acids (Collins and Wertz, 1985). The predicted amino acid sequences of both NS1 and NS2 do not provide obvious clues to their function. Using a minigenome replication system, the NS1 protein was shown to be a potent inhibitor of viral RNA transcription and replication (Atreya *et al.*, 1998). The NS2 protein was also suggested to be a transcriptional inhibitor (Atreya *et al.*, 1998). The M2-1 and M2-2 proteins are translated from a single M2 mRNA. The 22-kDa M2-1 protein is encoded by the 5′-proximal open reading frame of the M2 mRNA and partially overlaps the second M2-2 open reading frame

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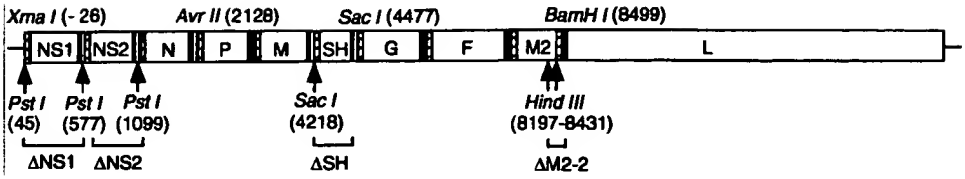

FIG. 1. Schematic diagram of full-length antigenomic cDNA. The positions at which the restriction enzyme sites were introduced to delete the corresponding genes are indicated.

(Collins and Wertz, 1985). The M2-1 protein has been shown to be involved in transcriptional processivity (Collins *et al.*, 1995). It decreases RNA transcriptional termination and facilitates readthrough of RNA transcription at gene junctions (Hardy and Wertz, 1998; Hardy *et al.*, 1999). The M2-2 protein encoded by the 3′-proximal open reading frame of the M2 mRNA, on the other hand, is a strong inhibitor of RSV RNA transcription and replication in a minigenome system (Collins *et al.*, 1996).

Recovery of infectious virus from cDNA has made it possible to manipulate the RSV genome and to study the functions of RSV genes with molecular approaches. Using the reverse genetics method, SH, NS2, and M2-2 have been reported to be dispensable for RSV replication *in vitro*. Although the recombinant RSV that lacked the SH gene replicated very well in tissue culture, the SH-deficient virus exhibited site-specific restriction in the respiratory tract of mice and was attenuated in chimpanzees (Bukreyev *et al.*, 1997; Whitehead *et al.*, 1999). The NS2 gene of both human and bovine RSV was dispensable for virus replication *in vitro*. However, NS2-minus RSV had small plaque morphology and exhibited reduced replication (Buchholz *et al.*, 1999; Teng and Collins, 1999). Recombinant RSV that lacked M2-2 protein expression was attenuated in tissue culture cells and in rodents (Jin *et al.*, 2000). ΔM2-2 virus produced significantly reduced levels of genomic RNA and antigenomic RNA, but the level of mRNA synthesis was not affected by the lack of M2-2 (Jin *et al.*, 2000; Bermingham and Collins, 1999). The data obtained so far suggest that M2-2 acts as a regulator during RNA synthesis, possibly by mediating the switch from transcription to genome replication to facilitate virus morphogenesis.

In this study, we generated a series of gene deletion mutants with deletions in NS1, NS2, SH, and M2-2 genes. We showed not only that NS1, NS2, SH, and M2-2 can be deleted from the RSV genome individually but also that two or more genes can be removed simultaneously from the RSV genome without affecting virus viability *in vitro*. However, all of the deletion mutants displayed altered phenotypes and showed reduced replication in tissue culture and in cotton rats. These results indicated that the accessory proteins encoded by RSV are important for virus replication.

RESULTS

Generation of various RSV gene deletion mutants

To examine the minimal gene components that are required for RSV replication *in vitro*, we used reverse genetics to delete those genes that are unique to RSV. NS1, NS2, SH, and M2-2 were removed from a RSV cDNA subclone through the introduced appropriate restriction enzyme sites. The cDNA fragments with the designed deletions were then introduced into the full-length RSV cDNA derived from the A2 strain (Fig. 1). The deletion of each gene from the antigenomic cDNA clone was confirmed by restriction enzyme digestion and by sequencing across the deletion junctions.

To recover recombinant RSV with the introduced deletions, the full-length RSV cDNA clone was transfected, together with plasmids encoding the RSV N, P, and L proteins under the control of the T7 promoter, into MVA-T7-infected HEp-2 cells. The culture supernatants from the transfected HEp-2 cells were used to infect fresh Vero cells to amplify the rescued viruses. Recovery of a deletion mutant was indicated by syncytial formation and confirmed by immunostaining of the infected cells using a polyclonal anti-RSV A2 serum. A total of 10 deletion mutants were obtained (Table 1); these included four single gene deletion mutants (ΔM2-2, ΔSH, ΔNS1, and ΔNS2) and six mutants with two or more genes deleted (ΔNS1NS2, ΔM2-2NS2, ΔM2-2SH, ΔSHNS1, ΔSHNS2,

TABLE 1

Recovery of RSV Deletion Mutants

cDNA construct	Genome length (nt)	Virus recovered
rA2	15222	+
ΔM2-2	14988	+
ΔSH	14964	+
ΔNS1	14690	+
ΔNS2	14699	+
ΔNS1NS2	14167	+
ΔM2-2NS2	14465	+
ΔM2-2SH	14730	+
ΔSHNS1	14432	+
ΔSHNS2	14441	+
ΔSHNS1NS2	13909	+
ΔM2-2NS1	14456	−
ΔM2-2SHNS1NS2	13675	−

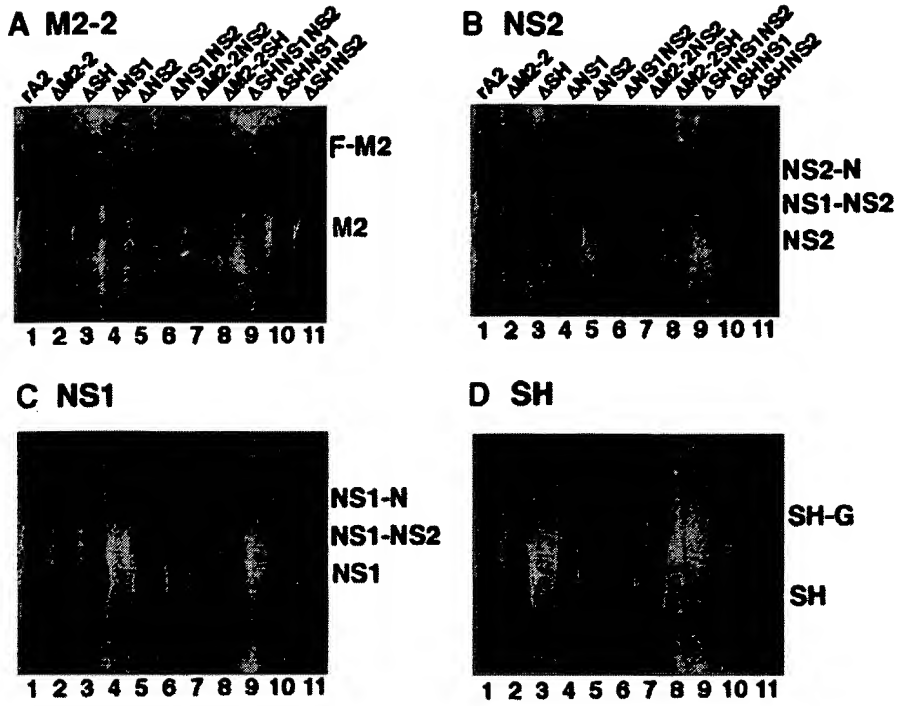

FIG. 2. Northern blot analysis of deletion mutants. Total intracellular RNAs isolated from virus-infected Vero cells were subjected to Northern blotting. Northern blots were hybridized with a digoxigenin-labeled riboprobe specific for the M2-2 (A), NS2 (B), NS1 (C), or SH (D). Monocistronic mRNA and polycistronic readthrough RNAs are indicated.

and ΔSHNS1NS2). ΔSHNS1NS2 had a deletion of 1313 nt and contained the shortest genome length among the viable viruses we were able to rescue. ΔM2-2NS1 was initially rescued in HEp-2 cells. RSV-like infectious foci were detected by immunostaining after the first round of passage in Vero cells. However, this virus was not amplified after subsequent passages in Vero cells. Thus the removal of both M2-2 and NS1 genes simultaneously severely debilitated the replication of RSV. Recovery of a recombinant RSV lacking the NS1, NS2, SH, and M2-2 genes also was not successful. We made an antigenomic cDNA that had the M2-1 open reading frame deleted but were not able to recover a recombinant RSV that lacked the M2-1 gene. All of the recovered deletion mutants were plaque purified three times and amplified in Vero cells. Deletions introduced into each mutant virus was confirmed by RT–PCR using pairs of primers spanning the deleted regions. Sequencing of the RT–PCR fragments further confirmed the presence of the deletions.

Northern blot analysis of mRNA

Viral RNA expression of the 10 viable deletion mutants was examined by Northern blot analysis. Total intracellular RNA was extracted from virus-infected Vero cells. RNA blots were hybridized with digoxigenin-labeled riboprobes that hybridized specifically to the NS1, NS2, SH, or M2-2 genes (Fig. 2). Northern blotting analysis further confirmed the presence of the various deletions in the mutant viruses. The NS1-NS2 readthrough RNA was absent from mutants lacking either NS1 or NS2 (Figs. 2B and 2C). It was noticed that viruses with a deletion of NS1 had increased levels of NS2 mRNA. Relatively more NS2 transcripts were produced in cells infected with ΔNS1 (lane 4) and ΔSHNS1 (lane 10).

Analysis of viral proteins

Western blotting and immunoprecipitation were used to analyze viral protein synthesis of the deletion mutants. Vero cells were infected with virus at an m.o.i. of 0.5. At 48 h postinfection, the infected cell lysates were harvested. Protein samples were separated by SDS–PAGE and analyzed by Western blotting using an anti-G monoclonal antibody, a polyclonal antibody that recognizes both the NS1 and NS2 proteins, or a polyclonal anti-SH antibody (Fig. 3A). As expected, deletion of the NS1 gene ablated expression of the NS1 protein for mutants ΔNS1, ΔNS1NS2, ΔSHNS1NS2, and ΔSHNS1. NS2 protein was not detected in cells infected with ΔNS2, ΔNS1NS2, ΔSHNS1NS2, and ΔSHNS2. The SH protein expression was abolished in cells infected with ΔSH, ΔM2-2SH, ΔSHNS1NS2, ΔSHNS1, and ΔSHNS2. Noticeably, much more NS2 protein was expressed in cells infected with viruses that had the NS1 gene deleted (ΔNS1, lane 4, and ΔSHNS1, lane 10), consistent with

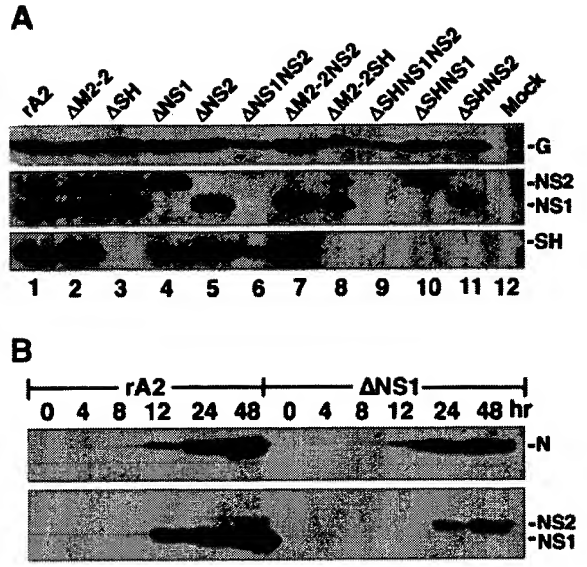

FIG. 3. Expression of RSV proteins. (A) The infected Vero cell extracts were harvested 48 h postinfection and subjected to Western blotting with antibodies against G, NS1/NS2, or SH. (B) Time course of NS1 and NS2 protein expression of rA2 and ΔNS1 viruses. The infected cell extracts were harvested at the indicated times of postinfection, and the Western blot was probed with anti-NS2 polyclonal antibody and anti-N monoclonal antibodies. The positions of viral proteins are indicated.

the RNA expression pattern. As a comparison, the G protein produced by ΔSH and ΔSHNS1 was comparable to that of wild-type rA2. Accumulation of NS2 protein in cells infected with ΔNS1 was compared with wild-type rA2 by Western blotting using anti-NS2 polyclonal antibody. NS1 was detected in rA2-infected cells at 8 h postinfection and was absent in ΔNS1-infected cells (Fig. 3B). NS2 was detected at 24 h postinfection, but the level of the NS2 protein synthesized in ΔNS1-infected cells was much greater than that of rA2. Replication of ΔNS1 was poorer than rA2 as judged by lower amount of the N protein accumulated in ΔNS1-infected cells (Fig. 3B, top). The protein expression pattern of ΔSHNS1 was similar to that of ΔNS1 (data not shown). The increased NS2 protein expression is most likely due to more NS2 mRNA synthesized in the infected cells (Fig. 2). Removal of the NS1 gene translocated the NS2 gene to the 5′ most proximal location, making NS2 the first RSV gene expressed. Expression of M2-2 protein by the 10 deletion mutants was examined through immunoprecipitation of the virus-infected cells by using a polyclonal antibody raised against M2-2 (Jin *et al.*, 2000). The M2-2 protein was not detected in cells infected with ΔM2-2, ΔM2-2SH, and ΔM2-2NS2 (data not shown). The pattern of viral protein expression of the 10 deletion mutants was compared with rA2 by immunoprecipitation. As shown in Fig. 4, the overall polypeptide patterns among viruses were very similar.

Replication of deletion mutants in tissue culture cells

Replication of all the deletion mutants was compared with wild-type rA2 in both HEp-2 and Vero cells. HEp-2 cells and Vero cells were infected with each mutant at an m.o.i. of 0.2 and incubated at 35°C for 3 days. Virus titers were quantified by plaque assay in Vero cells. As summarized in Table 2, all 10 deletion mutants showed reduced replication in Vero cells compared with rA2, with titer reductions ranging from 0.2-log for ΔM2-2 to 1.2-log for ΔNS1. The titer reduction obtained for ΔM2-2 in Vero cells was consistent with that reported previously under similar growth conditions (Jin *et al.*, 2000). In HEp-2 cells, a significant reduction in virus titer was observed for RSV mutants carrying the M2-2 deletion. For example, the deletion of M2-2 by itself resulted in a 2-log titer reduction compared with rA2. ΔM2-2NS2, which carried an additional NS2 deletion, was more restricted, exhibiting a 3-log reduction in HEp-2 cells. Unexpectedly, ΔM2-2SH, which had a deletion of SH as well as M2-2, had only an approximately 0.5-log reduction in HEp-2 cells. Mutants that contained NS1 and/or NS2 deletions had moderate levels of reduction in replication, approximately 1-log compared with wild-type rA2. ΔSH had a level of replication similar to that of rA2 in HEp-2 cells.

Plaque size for all of the deletion mutants was also compared with that of rA2 in these two cell lines. HEp-2 and Vero cells were infected with each deletion mutant or rA2 and incubated under an overlay containing 1× L15 medium and 1% methylcellulose for 6 days at 35°C. Plaques were immunostained with anti-RSV polyclonal antibodies, and plaque size was estimated from photographs of immunostained RSV plaques. As shown in Table 2, plaque size reduction was observed

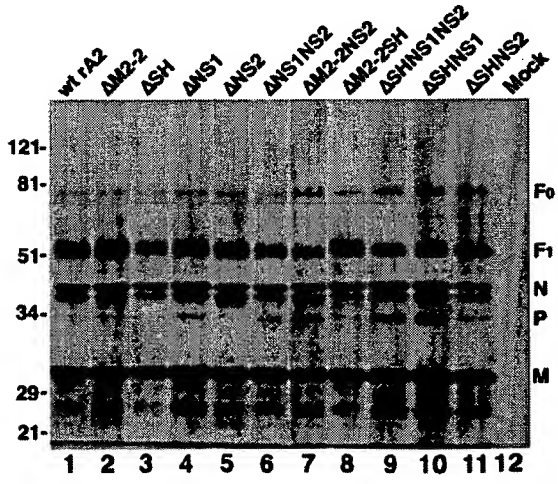

FIG. 4. Immunoprecipitation of viral proteins by anti-RSV antibody. RSV-infected Vero cells (m.o.i. = 0.5) were metabolically labeled with ^{35}S-ProMix (100 μCi/ml) for 4 h from 14 to 18 h postinfection. Cell lysates were immunoprecipitated with goat polyclonal anti-RSV antibody, separated on a 10% polyacrylamide gel, and processed for autoradiography. The positions of each viral protein are indicated on the right, and the molecular weight size markers are shown on the left.

TABLE 2

Replication of RSV Deletion Mutants *in Vitro* and *in Vivo*

Mutants	Replication[a]		Plaque size[b]		Replication in lungs of cotton rats[c] (Mean $\log_{10}$pfu/g ± SE)
	Vero	HEp-2	Vero	HEp-2	
rA2	6.7	6.2	100%	100%	4.2 ± 0.08
ΔM2-2	6.5	4.3	70%	30%	<2.0
ΔSH	6.3	6.0	90%	110%	3.8 ± 0.25
ΔNS1	5.5	5.5	80%	10%	2.2 ± 0.31
ΔNS2	6.0	5.5	90%	20%	2.2 ± 0.87
ΔNS1NS2	5.7	4.5	80%	5%	2.75 ± 0.10
ΔM2-2NS2	6.2	3.4	70%	<5%	<2.0
ΔM2-2SH	6.4	5.6	50%	30%	2.3 ± 1.2
ΔSHNS1	6.0	5.3	50%	5%	<2.0
ΔSHNS2	6.4	5.1	50%	10%	2.76 ± 0.06
ΔSHNS1NS2	6.3	5.1	50%	10%	<2.0

[a] HEp-2 or Vero cells were infected with mutants at m.o.i. of 0.2 for 3 days and levels of virus replication were determined by plaque assay in Vero cells.

[b] Vero and Hep-2 cells were infected with viruses as indicated and were incubated at 35°C for 6 days. Plaques were visualized by immunostaining and plaque size was an average of five measurements taken from photographed plaques.

[c] Cotton rats were infected with 2×10^5 pfu viruses intranasally and 4 days later, the lung tissues were homogenized and titrated for the amount of virus by plaque assay on Vero cells.

for all the deletion mutants in Vero cells, ranging from 10% to 50% compared with rA2. However, in HEp-2 cells, a greater reduction in plaque sizes was observed for most of the deletion mutants. ΔM2-2 and ΔM2-2SH had a modest reduction in plaque size (30% of that of rA2), whereas rA2ΔM2-2NS2 formed barely visible plaques in HEp-2 cells. Interestingly, ΔSH exhibited plaques that were slightly larger than those of rA2. The deletion of NS1 or NS2, or both, severely affected virus plaque formation in HEp-2 cells. The deletion of NS1 or NS2 reduced virus plaque size to 10–20% of that of rA2. Multiple gene deletion mutants, ΔSHNS1, ΔNS1NS2, ΔSHNS1, and ΔSHNS1NS2, had plaque sizes 5–10% of that of rA2.

Replication in cotton rats

All of the deletion mutants were evaluated for their ability to replicate in the lower respiratory tracts of cotton rats. Cotton rats in groups of five were inoculated with 10^5 pfu of virus intranasally. Four days postinoculation, animals were sacrificed, and their lung tissues were harvested and homogenized for virus titration in Vero cells. As summarized in Table 2, ΔSH showed a slight reduction in replication in the lungs of cotton rats. The replication of ΔNS1, ΔNS2, ΔNS1NS2, ΔM2-2SH, and ΔSHNS2 was attenuated by about 100-fold compared with that of rA2. ΔM2-2, ΔM2-2NS2, ΔSHNS1, and ΔSHNS1NS2 were very attenuated in cotton rats; the replication of these mutants in the lower respiratory tracts of cotton rats was below the limit of detection.

DISCUSSION

RSV has evolved to encode several unique proteins that are not shared by other members of the Paramyxoviridae family. The functions for these accessory proteins are not well defined. In this study, we demonstrated that not only can NS1, NS2, SH, and M2-2 be removed individually from RSV genome but also two or even three of these genes can be deleted from virus simultaneously in tissue culture. M2-2 can be deleted together with either SH or NS2; however, virus lacking both M2-2 and NS1 could not be recovered. Thus it is not possible to delete all four of these accessory genes from the RSV genome at the same time. Our attempts to recover RSV with M2-1 deletion have not been successful. M2-1 has been shown to be a transcription factor that increases transcription readthrough at gene junctions (Collins *et al.*, 1996; Hardy and Wertz, 1998; Hardy *et al.*, 1999). This function is essential for virus genome replication and transcription. Thus far, five RSV gene products have been found to be dispensable for virus replication *in vitro*; these include NS1 (the present study), NS2 (Teng and Collins, 1999; Buchholz *et al.*, 1999; the present study), SH (Bukreyev *et al.*, 1997; the present study), M2-2 (Bermingham and Collins, 1999; Jin *et al.*, 2000), and G (Karron *et al.*, 1997). It is remarkable that three genes can be deleted simultaneously without affecting virus viability in cell culture. However, all of the RSV mutants with one or more genes deleted exhibited various degrees of debilitated replication *in vitro* and *in vivo*.

When NS1, NS2, or M2-2 was deleted from RSV, the deletion mutants exhibited a greater defect in replication

in HEp-2 cells than in Vero cells. RSV surface glycoproteins are processed slightly differently in the human and simian cell lines (Rouledge *et al.,* 1986). However, Vero cells are also known to be deficient in the interferon (IFN) pathway (Desmyter *et al.,* 1968). Recently it was shown that many of the negative-strand RNA viruses possess an auxiliary IFN antagonist that allow them to circumvent cellular antiviral defense mechanisms. For example, the V protein of SV5 inhibits IFN signaling by inducing the degradation of STAT1, a component important for IFN signaling (Didcock *et al.,* 1999a,b). The Sendai virus C protein prevents the establishment of IFN-mediated antiviral response and appears to be a key determinant of Sendai virus pathogenicity (Garcin *et al.,* 1997, 1999). For the segmented negative-strand viruses, influenza virus NS1 protein was shown to act as a suppressor of PKR activation in virus-infected cells (Hatada *et al.,* 1999). A recombinant influenza virus containing a deletion of 38 amino acids from the N-terminus of NS1 grows to a high titer in IFN-deficient Vero cells, but its replication is impaired in MDCK cells. This $NS1^-$ virus is also pathogenic in $STAT1^{-/-}$ mice (Egorov *et al.,* 1998; Garcia-Sastre *et al.,* 1998). RSV has been found to be resistant to the antiviral effects mediated by human MxA and type 1 IFN (Atreya and Kulkarni, 1999). It is very likely that RSV also encodes a gene product or products that allow RSV replication to evade host-mediated antiviral responses. Further studies are needed to determine the role of RSV accessory gene products in viral pathogenesis.

The NS1 protein has been suggested to be an inhibitor of viral transcription and replication *in vitro* (Atreya *et al.,* 1998). This inhibition is seen at both the antigenomic and genomic promoters (Atreya *et al.,* 1998), but it is not known whether NS1 acts on the RNA template or on the polymerase components. The deletion of NS1 impaired virus replication, as indicated by its reduced plaque size and decreased virus yield. The interaction of NS1 with M has been demonstrated previously by coimmunoprecipitation of these two proteins with anti-NS1 or anti-M antibodies (Evans *et al.,* 1996). Because the M protein is involved in virus assembly, it is possible that NS1 may have a role in virion morphogenesis through its interaction with M; therefore, the lack of NS1 could cause inefficient virus budding.

The expression of RSV proteins is well regulated through transcription attenuation at each gene junction. NS2 is the second gene product expressed in the infected cells and is less abundant. When NS1 was deleted, the level of NS2 expression increased substantially. If NS2 is a negative transcription regulator, as suggested by *in vitro* studies (Atreya *et al.,* 1998), increased NS2 expression may alter transcription regulation in those mutants lacking the NS1 protein expression. Consistent with what has been described for the NS2 deletion mutant of both human and bovine strains (Teng and Collins, 1999; Buchholz *et al.,* 1999), the replication of ΔNS2 was decreased by approximately 10-fold compared with the wild-type recombinant RSV in both Vero and HEp-2 cells. ΔNS1NS2 was even more attenuated in HEp-2 cells, as indicated by 10-fold more titer reduction and much smaller plaque size compared with single NS1 or NS2 deletion mutants.

As reported previously (Bukreyev *et al.,* 1997), we also found that ΔSH has little effect on virus replication *in vitro*. The growth of ΔSH in tissue culture was similar or slightly better than that of the wild-type recombinant RSV. Among the 10 RSV deletion mutants examined, ΔSH is least attenuated in cotton rats according to its replication in the lower respiratory tracts of cotton rats. It has been reported that ΔSH is not attenuated in the upper respiratory tract but is moderately attenuated in the lower respiratory tract of chimpanzees, a host that is fully permissive to RSV infection (Whitehead *et al.,* 1999). Removal of the SH gene from ΔNS1, ΔNS2, ΔNS1NS2, and ΔM2-2 did not seem to further impair virus replication *in vitro*. On the contrary, the deletion of SH appeared to partially offset poor virus replication in HEp-2 cells that was caused by M2-2 deletion in ΔM2-2SH.

ΔM2-2 has altered replication in several human cell lines and is attenuated in mice and cotton rats (Jin *et al.,* 2000). M2-2 appears to be a regulator of virus genome replication and transcription (Jin *et al.,* 2000; Bermingham and Collins, 1999). Interestingly, large syncytial formation was observed for ΔM2-2 but not for ΔM2-2SH and ΔM2-2NS2 (data not shown). ΔM2-2 severely reduced the level of viral genomic and antigenomic RNA, possibly by altering the switch from mRNA synthesis to genome replication (Jin *et al.,* 2000; Bermingham and Collins, 1999). Similarly, greatly reduced genomic and antigenomic RNA synthesis was also observed for ΔM2-2SH and ΔM2-2NS2 (data not shown). ΔM2-2NS2 was very attenuated *in vitro*, as indicated by its low yield and poor plaque formation in infected HEp-2 cells. If both M2-2 and NS2 function as transcriptional regulators at different stages of RSV replication, their removal could have additive debilitating effects on virus replication. Although it was not difficult to obtain a virus with three genes deleted (ΔSHNS1NS2), it has not been possible to obtain a virus containing both M2-2 and NS1 deletions. The virus could not survive when both M2-2 and NS1 were deleted. It is possible that some of these accessory proteins interact with each other to regulate virus replication. We did not examine the replication of the deletion mutants in the upper respiratory tracts of cotton rats because our wild-type recombinant RSV replicates poorly in the nasal turbinates of cotton rats. Serious RSV disease is associated with virus replication in the lower respiratory tract. When inoculated intranasally into cotton rats, all of the deletion mutants exhibited varying degrees of attenuation in the lower respiratory tracts, indicating that accessory proteins of RSV may be critical for viral pathogenesis. An association of viral accessory

proteins with pathogenicity and virulence has been reported for other members of the paramyxoviruses (Kurotani *et al.*, 1998; Kato *et al.*, 1997a,b; Tober *et al.*, 1998). We plan to evaluate the replication of these deletion mutants in a more permissive host and to investigate them as potential vaccine candidates.

MATERIALS AND METHODS

Cells and viruses

Monolayer cultures of HEp-2 and Vero cells [American Type Culture Collection (ATCC), Rockville, MD] were maintained in minimal essential medium (MEM) containing 10% fetal bovine serum (FBS). Modified vaccinia virus Ankara (MVA-T7) expressing bacteriophage T7 RNA polymerase was provided by Dr. Bernard Moss and grown in CEK cells.

Construction of cDNA clones containing RSV gene deletions

To delete each RSV gene, appropriate restriction enzyme sites were introduced at each gene junction, and the protein-coding regions were removed from the antigenomic cDNA clone as described later (Fig. 1).

Construction of pA2ΔNS1. To delete the NS1 open reading frame from RSV, *Pst*I restriction enzyme sites were introduced at RSV nt 45 and nt 577 by site-directed mutagenesis (Quickchange mutagenesis kit; Qiagen, Studio City, CA). Mutagenesis was performed with pET(X/A) cDNA subclone containing the *Xba*I (26 nt)–*Avr*II (2128 nt) restriction fragment encoding the NS1, NS2, and part of the N gene of RSV. The 532-nt cDNA fragment containing the NS1 gene and the sequences encoding its gene start and gene end signal was removed by *Pst*I restriction enzyme digestion. The *Xba*I–*Avr*II restriction fragment with NS1 deletion was separated from the pET(X/A) vector and cloned into a full-length RSV cDNA clone (pA2) derived from RSV A2 strain (Jin *et al.*, 1998). The antigenomic cDNA clone contained a single nucleotide change at the fourth position (C to G in antigenomic sense) in the RSV leader region.

*Construction of pA2ΔNS2. Pst*I restriction enzyme sites were introduced at RSV nt 577 and nt 1099 in pET(X/A). Digestion of pET(X/A) plasmid containing the introduced *Pst*I restriction enzyme sites removed a 523-nt fragment of the NS2 gene, including its gene start and gene end sequences. The *Xba*I–*Avr*II restriction fragment with NS2 deletion was cloned into pA2 and was designated pA2ΔNS2.

Construction of pA2ΔSH. An *Sac*I restriction enzyme site was introduced in the gene start sequence of the SH gene (nt 4219) in a cDNA subclone pET(A/S) that contained RSV sequence from nt 2128 (*Avr*II) to nt 4477 (*Sac*I). A unique *Sac*I site (nt 4477) was present in the viral sequence at the end of the SH open reading frame. Digestion with *Sac*I allowed the removal of a 257-nt fragment containing the gene start sequence and the entire open reading frame of the SH gene but not the gene stop sequence. The *Avr*II–*Sac*I restriction fragment with SH deletion was then cloned into pA2 and was designated pA2ΔSH.

Construction of pA2ΔM2-2. The M2-2 open reading frame was largely deleted from pA2 through the introduced *Hin*dIII restriction enzyme sites as previously described (Jin *et al.*, 2000).

Construction of pA2ΔNS1NS2. The NS1 and NS2 genes were deleted from RSV cDNA subclone pET(X/A) through the introduced *Pst*I sites at RSV nt 45 and nt 1100. The *Xba*I–*Avr*II restriction fragment with a deletion of 1055 nt was cloned into pA2, and the plasmid was designated as pA2ΔNS1NS2.

Construction of pA2ΔM2-2SH. The SH and M2-2 genes were deleted from the full-length RSV cDNA clone by using existing unique restriction sites. The *Sac*I–*Bam*HI fragment with M2-2 deletion in pET(S/B) subclone was removed by digestion with *Sac*I and *Bam*HI restriction enzymes and ligated with the full-length RSV antigenomic cDNA clone containing the SH gene deletion (pA2ΔSH). The resulting plasmid with deletions of both SH and M2-2 was designated pA2ΔM2-2SH.

Construction of pA2ΔM2-2NS2. To delete both M2-2 and NS2 from the full-length RSV cDNA clone, the *Xba*I–*Avr*II fragment containing a deletion of NS2 in pET(X/A) subclone was introduced into the full-length RSV antigenomic cDNA clone with the M2-2 gene deletion (pA2ΔM2-2). The resulting plasmid with deletions of both M2-2 and NS2 was designated pA2ΔM2-2NS2.

Construction of pA2ΔSHNS1. To delete both SH and NS1 from the full-length RSV cDNA clone, the *Xba*I–*Avr*II fragment containing a deletion of NS1 was introduced into pA2ΔSH. The resulting plasmid with deletions of both SH and NS1 was designated pA2ΔSHNS1.

Construction of pA2ΔSHNS2. To delete both SH and NS2 from the full-length RSV cDNA clone, the *Xba*I–*Avr*II fragment with NS2 deletion was introduced into pA2ΔSH. The obtained plasmid with deletions of both SH and NS2 was designated pA2ΔSHNS2.

Construction of pA2ΔSHNS1NS2. To delete three RSV genes (SH, NS1, and NS2) simultaneously from the full-length RSV antigenomic cDNA clone, the *Xba*I–*Avr*II fragment with deletions of NS1 and NS2 was introduced into pA2ΔSH. The plasmid with SH, NS1, and NS2 deleted was designated pA2ΔSHNS1NS2.

Construction of pA2ΔM2-2NS1. The *Xba*I–*Avr*II fragment containing a deletion of NS1 was introduced into pA2ΔM2-2. The antigenomic cDNA containing deletions of both M2-2 and NS1 was designated pA2ΔM2-2NS1.

Construction of pA2ΔM2-2SHNS1NS2. The *Xba*I–*Avr*II fragment with both NS1 and NS2 deleted was introduced into pA2ΔM2-2SH. The obtained antigenomic cDNA contained deletions of four RSV genes (NS1, NS2, SH, and

M2-2) in a RSV A2 backbone and was designated pA2ΔM2-2SHNS1NS2.

The cDNA fragments derived from plasmids that have undergone mutagenesis reactions were sequenced to ensure that no other mutations were introduced incidentally. Each full-length antigenomic cDNA clone containing the designed deletions was confirmed by restriction enzyme digestion and by sequencing across the junction of the deleted region.

Recovery of RSV gene deletion mutants

Recovery of RSV gene deletion mutants was performed as described previously (Jin *et al.*, 1998, 2000). Infectious viruses were rescued from the transfected HEp-2 cells and were amplified by one passage in Vero cells before plaque purification. Each recombinant RSV was plaque-purified three times and grown in Vero cells. Virus titer was determined by plaque assay, which was enumerated by immunostaining with an anti-RSV antibody (Biogenesis, Sandown, NH).

Northern blotting analysis

For Northern blot hybridization analysis, Vero cells in 6-well plates were separately infected with various RSV gene deletion mutants and rA2 at an m.o.i. of 0.5 and incubated at 35°C for 24 h. Total cellular RNA was extracted from the infected cell monolayers by using an RNA extraction kit (RNA STAT-60; Tel-Test, Friendswood, TX). RNA obtained from each well was divided into four aliquots and electrophoresed on 1.2% agarose gels containing formaldehyde in quadruplicate. The RNAs were then transferred to nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ), and each blot was hybridized with a digoxigenin-labeled riboprobe specific for NS1, NS2, SH, or M2-2. RNAs that hybridized with riboprobes were visualized using Dig-Luminescent Detection Kit for Nucleic Acids (Boehringer-Mannheim, Indianapolis, IN).

Protein expression analysis by Western blotting and immunoprecipitation

Western blotting was used to monitor viral protein expression of the deletion mutants. Vero cells in 6-well plates were infected with each virus at an m.o.i. of 0.5. After 48 h of infection, the infected cells were lysed in protein lysis buffer. The cell lysates were electrophoresed on 17.5% polyacrylamide gels containing 0.1% SDS and 4 M urea, and the proteins were transferred to nylon membranes. One blot was hybridized with a polyclonal antiserum raised against the C-terminal 12 amino acids of NS2. This antiserum recognizes both the NS1 and NS2 proteins because the C-terminal four residues are shared by both proteins (Evans *et al.*, 1996). The other blots were probed with a polyclonal anti-SH antibody or a monoclonal antibody against the G protein. Western blotting was performed as described previously (Jin *et al.*, 1996).

For immunoprecipitation analysis, Vero cells were infected with each virus at an m.o.i. of 0.5 and labeled with ^{35}S-ProMix (100 μCi/ml ^{35}S-Cys and ^{35}S-Met; Amersham, Arlington Heights, IL) from 14 to 18 h postinfection. The labeled cell monolayers were lysed with RIPA buffer, and the polypeptides were immunoprecipitated with a polyclonal anti-RSV A2 serum (Biogenesis). The immunoprecipitated polypeptides were electrophoresed on a 10% polyacrylamide gel containing 0.1% SDS and detected by autoradiography.

Replication of recombinant RSV deletion mutants in tissue culture

Plaque formation of each deletion mutant was examined by plaque assay in HEp-2 and Vero cells using an overlay composed of 1% methylcellulose and 1× L15. The infected cells were incubated at 35°C for 6 days, and the plaques were enumerated by immunostaining using a polyclonal antiserum against RSV. The plaque size for each mutant was estimated from photographed microscopic images. The mean size was averaged from five plaques and compared with the averaged size of the wild-type rA2 plaque. To compare replication of RSV deletion mutants with wild-type rA2 in tissue culture cells, Vero or HEp-2 cells were infected with each virus at an m.o.i. of 0.2 and incubated at 35°C for 3 days. The amount of virus released from the infected cells was determined by plaque assay in Vero cells.

Virus replication in cotton rats

Each deletion mutant was compared with the wild-type rA2 for its ability to replicate in the lower respiratory tracts of cotton rats. Four- to 6-week-old *S. hispidus* cotton rats that were free of respiratory pathogens (Virion Systems, Rockville, MD) were inoculated in groups of five intranasally under light methoxyflurane anesthesia with 10^5 pfu in a 0.1-ml inoculum of rA2 or mutant viruses. On day 4 postinoculation, animals were sacrificed by CO_2 asphyxiation, and their lungs were obtained separately. Tissues were homogenized in 2 ml of OptiMEM, and virus titers were determined by plaque assay in Vero cells.

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Respiratory Syncytial Virus That Lacks Open Reading Frame 2 of the M2 Gene (M2-2) Has Altered Growth Characteristics and Is Attenuated in Rodents

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The M2 gene of respiratory syncytial virus (RSV) encodes two putative proteins: M2-1 and M2-2; both are believed to be involved in the RNA transcription or replication process. To understand the function of the M2-2 protein in virus replication, we deleted the majority of the M2-2 open reading frame from an infectious cDNA clone derived from the human RSV A2 strain. Transfection of HEp-2 cells with the cDNA clone containing the M2-2 deletion, together with plasmids that encoded the RSV N, P, and L proteins, produced a recombinant RSV that lacked the M2-2 protein (rA2ΔM2-2). Recombinant virus rA2ΔM2-2 was recovered and characterized. The levels of viral mRNA expression for 10 RSV genes examined were unchanged in cells infected with rA2ΔM2-2, except that a shorter M2 mRNA was detected. However, the ratio of viral genomic or antigenomic RNA to mRNA was reduced in rA2ΔM2-2-infected cells. By use of an antibody directed against the bacterially expressed M2-2 protein, the putative M2-2 protein was detected in cells infected with wild-type RSV but not in cells infected with rA2ΔM2-2. rA2ΔM2-2 displayed a small-plaque morphology and grew much more slowly than wild-type RSV in HEp-2 cells. In infected Vero cells, rA2ΔM2-2 exhibited very large syncytium formation compared to that of wild-type recombinant RSV. rA2ΔM2-2 appeared to be a host range mutant, since it replicated poorly in HEp-2, HeLa, and MRC5 cells but replicated efficiently in Vero and LLC-MK2 cells. Replication of rA2ΔM2-2 in the upper and lower respiratory tracts of mice and cotton rats was highly restricted. Despite its attenuated replication in rodents, rA2ΔM2-2 was able to provide protection against challenge with wild-type RSV A2. The genotype and phenotype of the M2-2 deletion mutant were stably maintained after extensive in vitro passages. The attenuated phenotype of rA2ΔM2-2 suggested that rA2ΔM2-2 may be a potential candidate for use as a live attenuated vaccine.

Human respiratory syncytial virus (RSV) has been recognized as a major infectious etiologic agent of pediatric respiratory tract diseases worldwide. RSV is the prototype member of the *Pneumovirus* genus of the *Paramyxoviridae* family (23). The RSV genome is a single-stranded negative-sense RNA of 15,222 nucleotides (nt) and encodes 11 proteins: NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L. The nucleoprotein (N protein), the phosphoprotein (P protein), and the major polymerase protein (L protein) are associated with the viral RNA genome in the form of nucleocapsids. The N, P, and L proteins form the viral RNA-dependent RNA polymerase complex for transcription and replication of the RSV genome (13, 33). The G and F proteins are the major integral surface glycoproteins involved in virus entry into cells. The matrix protein (M protein) is a peripheral membrane protein located between viral nucleocapsids and the viral envelope. The small hydrophobic protein (SH protein) is also membrane associated and has counterparts only in the rubulaviruses SV5 (16, 18) and mumps virus (11). Recombinant RSV lacking the SH protein gene replicates very well in tissue cultures, demonstrating that the SH protein is a nonessential protein (3). The NS1, NS2, M2-1, and M2-2 proteins lack known counterparts in other paramyxoviruses. The NS1 and NS2 proteins are nonstructural proteins, and the NS1 protein has been shown to be a potent viral RNA transcription and replication inhibitor (1). Recent work has shown that the NS2 gene is also dispensable for RSV replication in vitro, but small-plaque morphology and reduced replication were observed for the virus lacking the NS2 gene (2, 28).

The RSV M2 gene is located between the genes encoding the F and L proteins and encodes two putative proteins: M2-1 and M2-2. The 22-kDa M2-1 protein is encoded by the 5′-proximal open reading frame of the M2 mRNA, and its open reading frame partially overlaps the second, M2-2, open reading frame by a sequence encoding 10 amino acids (10). The M2-1 protein has been shown to be a transcriptional processivity factor that is involved in RNA transcription elongation (9). The M2-1 protein also decreases RNA transcription termination and facilitates read-through of RNA transcription at each gene junction (14, 15). The predicted M2-2 polypeptide contains 90 amino acids, but the M2-2 protein has not yet been identified intracellularly (10). The M2-2 protein down-regulates RSV RNA transcription and replication in a minigenome model system (9). The significance of this negative effect on RSV RNA transcription and replication in the viral replication cycle is not known.

To examine the function of the M2-2 protein, we generated a recombinant RSV that no longer expresses the M2-2 protein by using a recently developed reverse-genetics system (8, 19). Virus recovery was obtained by cotransfecting the RSV antigenomic cDNA that had the M2-2 open reading frame largely deleted, together with plasmids encoding the N, P, and L proteins, into cells that were infected concomitantly with a recombinant vaccinia virus expressing the T7 RNA polymerase. Viable RSV that lacked M2-2 protein expression was obtained, but it displayed altered growth phenotypes in tissue culture cells and was attenuated in rodent hosts. Our data suggested that the M2-2 protein, although dispensable for virus

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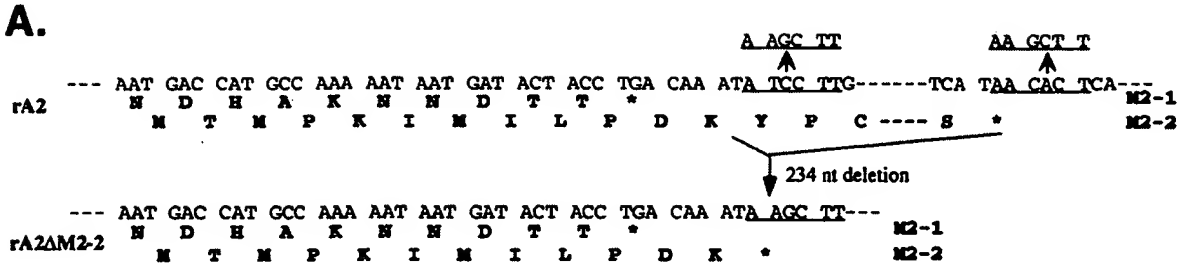

FIG. 1. Structure of the rA2ΔM2-2 genome and recovery of rA2ΔM2-2. (A) Sequences of the M2 gene in which the M2-1 and M2-2 open reading frames overlap. A total of 234 nt encoding the C-terminal 78 amino acids of the M2-2 protein were deleted through the introduced *Hin*dIII sites (underlined). The N-terminal 12 amino acid residues encoded by the M2-2 open reading frame were maintained at the region of overlap with the M2-1 open reading frame. (B) RT-PCR products of rA2ΔM2-2 and rA2 RNAs, obtained with a pair of primers flanking the M2 gene in the presence (+) or absence (−) of reverse transcriptase (RT). The size (in base pairs) of the DNA product derived from rA2 or rA2ΔM2-2 is indicated. The left lane was loaded with a 100-bp DNA size marker.

replication, plays an important role in virus infection and pathogenesis in vivo.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of HEp-2, HeLa, MDBK, LLC-MK2, and Vero cells (obtained from the American Type Culture Collection [ATCC]) were maintained in minimal essential medium containing 10% fetal bovine serum (FBS). MRC5 cells (obtained from the ATCC) were maintained in Dulbecco's modified Eagle medium containing 10% FBS. HEp-2 cells were obtained at passage level 362 and were not used beyond passage level 375. All the other cell lines were used within 20 in vitro passages. Modified vaccinia virus Ankara (MVA-T7) expressing bacteriophage T7 RNA polymerase (26, 32) was provided by Bernard Moss and grown in CEK cells.

Production of polyclonal antibody against the M2-2 protein. To produce antiserum against the M2-2 protein of RSV, a cDNA fragment encoding the M2-2 open reading frame from nt 8155 to nt 8430 was amplified by PCR and cloned into the pRSETA vector (Invitrogen, Carlsbad, Calif.). The resulting construct, pRSETA/M2-2, was transformed into BL21-Gold(DE3)plysS cells (Strategene, La Jolla, Calif.), and the expression of the His-tagged M2-2 protein was induced by isopropyl-β-D-thiogalactopyranoside (IPTG). The M2-2 fusion protein was purified through HiTrap affinity columns (Amersham Pharmacia Biotech, Piscataway, N.J.) and was used to immunize rabbits. Two weeks after a booster immunization, rabbits were bled and the serum was collected.

Construction of an M2-2 deletion cDNA. To generate an RSV antigenomic cDNA with an M2-2 deletion (pA2ΔM2-2), a cDNA fragment of 234 nt that contained the majority of the C-terminal part of the M2-2 open reading frame was removed from an antigenomic cDNA clone. The sequence encoding the N-terminal 12 amino acids of the M2-2 open reading frame that mostly overlaps the M2-1 open reading frame was maintained. A two-step cloning procedure was performed to delete the M2-2 open reading frame. Two *Hin*dIII restriction enzyme sites were introduced at RSV nt 8197 and nt 8431 in a cDNA subclone (pET-S/B) that contained the RSV *Sac*I (nt 4477)-*Bam*HI (nt 8499) cDNA fragment by use of a Quickchange mutagenesis kit (Strategene). Digestion of this cDNA subclone with the *Hin*dIII restriction enzyme removed the 234-nt *Hin*dIII cDNA fragment that contained the majority of the M2-2 open reading frame, and the remaining *Sac*I-*Bam*HI fragment with the M2-2 deletion was then cloned into an RSV antigenomic cDNA clone that contained a C to G change at the fourth position of the leader sequence, pRSVC4G (19). The resulting plasmid was designated pA2ΔM2-2 (Fig. 1).

Recovery of rA2ΔM2-2. Recombinant RSV was recovered from cDNA as described by Jin et al. (19). Briefly, HEp-2 cells at 80% confluence in a six-well plate were infected with MVA-T7 at a multiplicity of infection (MOI) of 5 PFU/cell for 1 h and then were transfected with plasmids encoding the RSV N, P, and L proteins and pA2ΔM2-2 by use of LipofecTACE (Life Technologies, Gaithersburg, Md.). After 5 h of incubation of the transfected HEp-2 cells at 35°C, the medium was replaced with minimal essential medium containing 2% FBS, and the cells were further incubated at 35°C for 3 days. The rescued virus (rA2ΔM2-2 [recombinant RSV that lacked the M2-2 open reading frame]) recovered from the transfected cells was plaque purified three times and amplified in Vero cells. The virus titer was determined by a plaque assay, and plaques were visualized by immunostaining with polyclonal anti-RSV A2 serum (Biogenesis, Sandown, N.H.).

Growth analysis of recombinant RSV in tissue cultures. To compare the plaque morphology of rA2ΔM2-2 with that of recombinant RSV A2 (rA2), HEp-2 or Vero cells were infected with each virus and overlaid with semisolid medium composed of 1% methylcellulose and L15 medium (JRH Biosciences, Lenexa, Kans.) with 2% FBS. Five days after infection, infected cells were immunostained with antisera against the RSV A2 strain. Plaque size was determined by measuring plaques from photographed microscopic images. A growth cycle analysis of rA2ΔM2-2 in comparison with rA2 was performed with both HEp-2 and Vero cells. Cells grown in 6-cm dishes were infected with rA2 or rA2ΔM2-2 at an MOI of 0.5. After 1 h of adsorption at room temperature, infected cells were washed three times with phosphate-buffered saline, the medium was replaced with 4 ml of OptiMEM (Life Technologies), and the culture was incubated at 35°C in an incubator containing 5% CO_2. At various times

postinfection, 200 μl of culture supernatant was collected and stored at −70°C until virus titration. Each aliquot taken was replaced with an equal amount of fresh medium. The virus titer was determined by a plaque assay on Vero cells, using an overlay of 1% methylcellulose and L15 medium containing 2% FBS. To analyze virus replication in different host cells, each cell line grown in six-well plates was infected with rA2ΔM2-2 or rA2 at an MOI of 0.2. Three days postinfection, the culture supernatants were collected, and virus was quantitated by a plaque assay on Vero cells.

RNA extraction, RT-PCR, and Northern blot analysis. For reverse transcription (RT)-PCR, viral RNA was extracted from rA2ΔM2-2- and rA2-infected cell culture supernatants by use of an RNA extraction kit (RNA STAT-50; Tel-Test, Friendswood, Tex.). Viral RNA was reverse transcribed with reverse transcriptase and a primer complementary to the viral genome from nt 7430 to nt 7449. The cDNA fragment spanning the M2 gene was amplified by PCR with primer V1948 (nt 7486 to nt 7515 for positive sense) and primer V1581 (nt 8544 to nt 8525 for negative sense). The PCR product was analyzed on a 1.2% agarose gel and visualized by ethidium bromide staining.

For Northern blot hybridization analysis, total cellular RNA was extracted from rA2ΔM2-2- or rA2-infected cells by use of an RNA extraction kit (RNA STAT-60; Tel-Test). RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde and transferred to a nylon membrane (Amersham Pharmacia Biotech). The membrane was hybridized with an RSV gene-specific riboprobe labeled with digoxigenin. The hybridized RNA bands were visualized by use of a Dig-Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). To detect viral genomic RNA, a ^{32}P-labeled riboprobe specific for the negative-sense F gene or N gene was used in Northern blot hybridization. To detect viral antigenomic RNA and mRNA, a ^{32}P-labeled riboprobe specific for the positive-sense F gene or G gene was used. Hybridization of the membrane with riboprobes was done at 65°C. Membrane washing and signal detection were performed according to standard procedures.

Immunoprecipitation and Western blotting of viral polypeptides. Virus-specific proteins produced from infected cells were analyzed by immunoprecipitation of the infected-cell extracts or by Western blotting. For immunoprecipitation analysis, Vero cells were infected with virus at an MOI of 1.0 and labeled with ^{35}S-promix (100 μCi each of [^{35}S]Cys and [^{35}S]Met per ml; Amersham, Arlington Heights, Ill.) at 14 to 18 h postinfection. The labeled cell monolayers were lysed with radioimmunoprecipitation assay buffer, and the polypeptides were immunoprecipitated with polyclonal anti-RSV A2 serum (Biogenesis) or anti-M2-2 protein antiserum. Immunoprecipitated polypeptides were electrophoresed on 17.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 4 M urea and detected by autoradiography. For Western blotting analysis, HEp-2 and Vero cells were infected with rA2ΔM2-2 or rA2. At various times postinfection, virus-infected cells were lysed in protein lysis buffer, and the cell lysates were electrophoresed on 17.5% polyacrylamide gels containing 0.1% sodium dodecyl sulfate and 4 M urea. The proteins were transferred to a nylon membrane. Immunoblotting was performed as described in Jin et al. (20) with polyclonal antiserum against M2-1 protein (gift of Jayesh Meanger), NS1 protein, or SH protein (gift of Jose A. Melero).

Virus replication in mice and cotton rats. Virus replication in vivo was determined with respiratory-tract-pathogen-free 12-week-old BALB/c mice (Simonsen Laboratories, Gilroy, Calif.) and *S. hispidus* cotton rats (Virion Systems, Rockville, Md.). Mice or cotton rats in groups of six were inoculated intranasally under light methoxyflurane anesthesia with 10^6 PFU of rA2 or rA2ΔM2-2 in a 0.1-ml inoculum per animal. On day 4 postinoculation, animals were sacrificed by CO_2 asphyxiation, and their nasal turbinates and lungs were obtained separately. Tissues were homogenized, and virus titers were determined by a plaque assay on Vero cells. To evaluate immunogenicity and protective efficacy, three groups of mice were inoculated intranasally with rA2, rA2ΔM2-2, or medium only at day 0. Three weeks later, mice were anesthetized, serum samples were collected, and a challenge inoculation of 10^6 PFU of biologically derived wild-type RSV A2 was administered intranasally. Four days postchallenge, the animals were sacrificed, both nasal turbinates and lungs were harvested, and virus titers were determined by a plaque assay. Serum neutralizing antibodies against RSV A2 were determined by a 60% plaque reduction assay (7) and by immunostaining of RSV-infected cells.

RESULTS

Generation of rA2ΔM2-2. Previously, we reported the recovery of recombinant RSV from an infectious cDNA clone derived from RSV strain A2 (19). To obtain recombinant RSV in which the expression of the M2-2 open reading frame is ablated, a 234-nt cDNA fragment that encodes the C-terminal 78 amino acids of the M2-2 protein was deleted from the infectious RSV cDNA clone. The N-terminal 12 amino acids that mostly overlapped with the M2-1 open reading frame were maintained, as it was considered likely that these 12 amino acids would not be sufficient to preserve M2-2 protein function (Fig. 1A). The deletion of the M2-2 open reading frame in antigenomic cDNA was confirmed by restriction enzyme digestion and by sequencing across the junction of the deletion. The resulting antigenomic cDNA clone, pA2ΔM2-2, is 14,988 nt long, 234 nt shorter than pRSVC4G.

Since pA2ΔM2-2 was not completely sequenced, two independent clones were obtained and used in the recovery of infectious virus. To recover recombinant RSV with the M2-2 open reading frame largely deleted, pA2ΔM2-2 was transfected, together with plasmids encoding the RSV N, P, and L proteins, under the control of the T7 promoter, into HEp-2 cells which had been infected with a modified vaccinia virus expressing the T7 RNA polymerase (MVA-T7). Culture supernatants from the transfected HEp-2 cells were used to infect fresh HEp-2 or Vero cells to amplify the rescued virus. The recovery of rA2ΔM2-2 was indicated by syncytium formation and confirmed by positive staining of infected cells with polyclonal anti-RSV A2 serum. Recovered rA2ΔM2-2 was plaque purified three times and amplified in Vero cells. To confirm that rA2ΔM2-2 contained the M2-2 deletion, viral RNA was extracted from virus and subjected to RT-PCR with a pair of primers spanning the M2 gene. As shown in Fig. 1B, rA2 yielded a PCR DNA product corresponding to the predicted 1,029-nt fragment, whereas rA2ΔM2-2 yielded a PCR product of 795 nt, 234 nt shorter. Generation of the RT-PCR product was dependent on the RT step, indicating that the product was derived from RNA rather than from DNA contamination. The deletion was also confirmed by sequencing analysis of the 795-nt RT-PCR DNA product derived from rA2ΔM2-2.

Replication of rA2ΔM2-2 in tissue culture cells. Plaque formation of rA2ΔM2-2 in HEp-2 and Vero cells was compared with that of rA2. As shown in Fig. 2A, rA2ΔM2-2 formed very small plaques in HEp-2 cells, with a reduction in virus plaque size of about fivefold observed for rA2ΔM2-2 compared to rA2. However, only a slight reduction in plaque size (30%) was seen in Vero cells infected with rA2ΔM2-2. In infected Vero cells, rA2ΔM2-2 formed very large syncytia compared to rA2 (Fig. 2B). Increased syncytium formation was not observed in HEp-2 cells. The growth cycle of rA2ΔM2-2 was also compared with that of rA2 in both HEp-2 and Vero cells (Fig. 3). In HEp-2 cells, rA2ΔM2-2 showed very slow growth kinetics, and the peak titer of rA2ΔM2-2 was about 2.0 log units lower than that of rA2. In Vero cells, rA2ΔM2-2 reached a peak titer similar to that of rA2. rA2ΔM2-2 was further examined for its growth properties in various cell lines derived from different hosts with different tissue origins (Table 1). Significantly reduced replication of rA2ΔM2-2, about 2 orders of magnitude less than that of rA2, was observed in infected HEp-2, MRC-5, and HeLa cells, all of human origin. However, the replication of rA2ΔM2-2 was only slightly reduced in MDBK and LLC-MK2 cells, which are derived from bovine and rhesus monkey kidney cells, respectively. It is known that HEp-2 cells from the ATCC contain HeLa cell markers; thus, HEp-2 cells may behave like HeLa cells.

rA2ΔM2-2 mRNA synthesis. To examine mRNA synthesis from rA2ΔM2-2 and rA2, the accumulation of M2 mRNA and the other viral mRNA products in infected Vero cells was analyzed by Northern blot hybridization. Hybridization of the blot with a riboprobe specific for the M2-2 open reading frame did not reveal any signal in rA2ΔM2-2-infected cells. Instead, a short M2 mRNA was detected in rA2ΔM2-2-infected cells by a riboprobe specific for the M2-1 open reading frame (Fig. 4A). These observations confirmed that the M2-2 open reading frame was deleted from rA2ΔM2-2. The accumulation of the other nine RSV mRNA transcripts was also examined, and the amounts of each mRNA were found to be comparable between

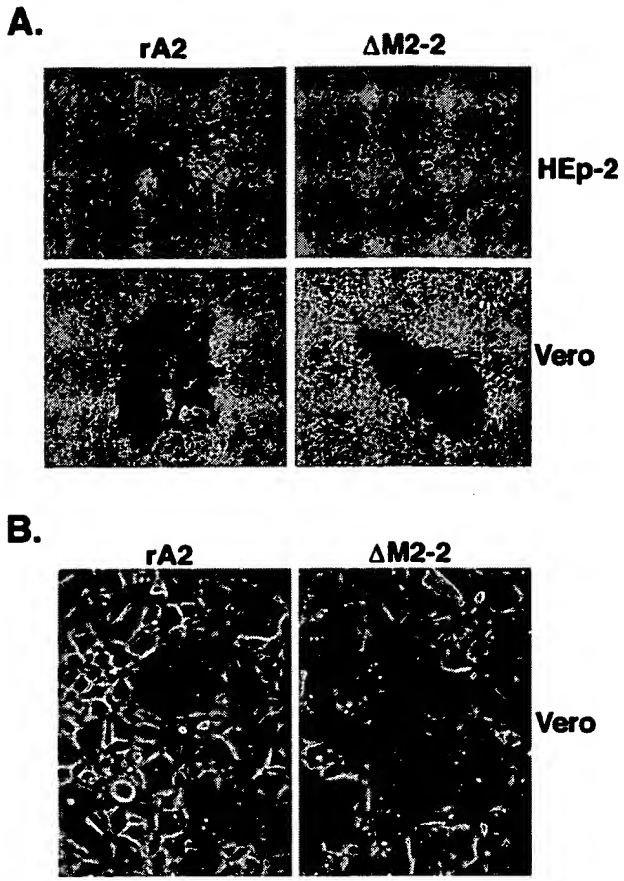

FIG. 2. Comparison of the abilities of rA2 and rA2ΔM2-2 to form plaques and syncytia. (A) Plaque morphology of rA2ΔM2-2 and rA2. HEp-2 or Vero cells were infected with rA2ΔM2-2 or rA2 under a semisolid overlay composed of 1% methylcellulose and L15 medium containing 2% FBS for 5 days. Virus plaques were visualized by immunostaining with a goat polyclonal anti-RSV antiserum and photographed under a microscope. (B) Comparison of syncytium formation. Vero cells were infected with rA2 and rA2ΔM2-2 at an MOI of 0.5 and incubated in liquid medium (OptiMEM) at 35°C for 40 h. The infected cell monolayers were photographed without any treatment.

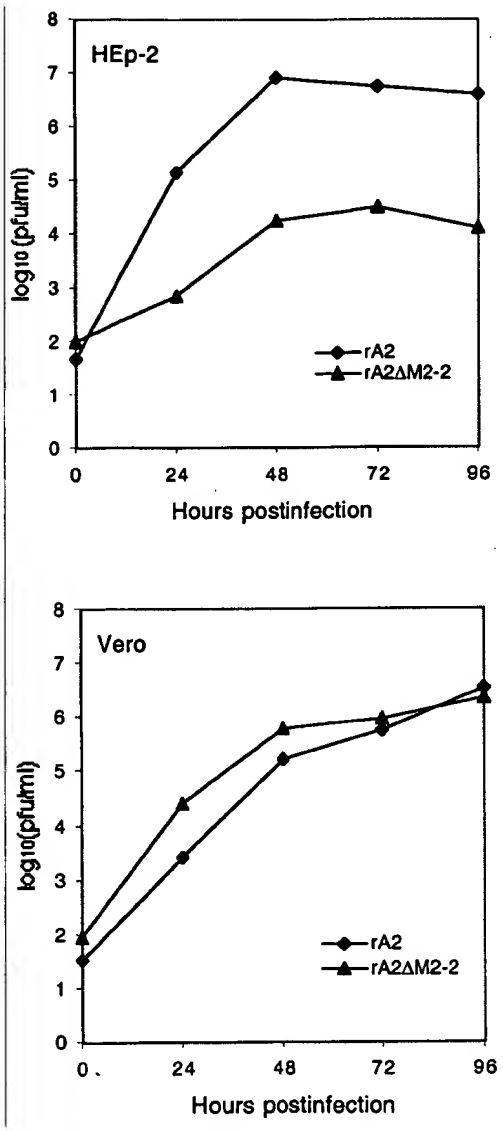

FIG. 3. Growth curves of rA2ΔM2-2 in HEp-2 and Vero cells. Vero cells or HEp-2 cells were infected with rA2ΔM2-2 or rA2 at an MOI of 0.5, and aliquots of medium were harvested at 24-h intervals. The virus titers were determined by a plaque assay on Vero cells. The virus titer at each time point is an average from two experiments with two independent isolates for both viruses.

rA2ΔM2-2- and rA2-infected cells. Examples of Northern blots probed with riboprobes specific for the N, SH, G, or F genes are also shown in Fig. 4A. Slightly faster migration of F-M2 bicistronic mRNA was also discernible due to the deletion of the M2-2 open reading frame.

The M2-2 protein was previously reported to be a potent transcriptional negative regulator in a minigenome replication assay. However, the lack of M2-2 protein expression did not appear to affect viral mRNA production in infected cells. To determine if the levels of viral antigenomic and genomic RNAs of rA2ΔM2-2 were affected by the M2-2 deletion, we examined the amounts of viral genomic and antigenomic RNAs produced in infected Vero and HEp-2 cells by Northern hybridization. Hybridization of the infected total cellular RNA with a ^{32}P-labeled F or N gene riboprobe specific for the negative-sense genomic RNA indicated that much less genomic RNA was produced in cells infected with rA2ΔM2-2 than in cells infected with rA2 (Fig. 4B). A duplicate membrane was hybridized with a ^{32}P-labeled F or G gene riboprobe specific for the positive-sense RNA. Very little antigenomic RNA was detected in cells infected with rA2ΔM2-2; the amount of the F or G mRNA in rA2ΔM2-2-infected cells was comparable to that in rA2-infected cells. It is very striking that the levels of both genomic and antigenomic RNAs in rA2ΔM2-2-infected cells were significantly reduced. Quantitation of the ratio of genomic and antigenomic RNA amounts to the viral mRNA amount indicated that at least a 10-fold reduction in antigenomic and genomic RNA amounts was observed in rA2ΔM2-2-infected cells. Therefore, it appears that RSV genome and antigenome syntheses were down-regulated due to the M2-2 deletion. This down-regulation was seen in both Vero and HEp-2 cells and thus was not cell type dependent. This phenomenon has been observed with different riboprobes and two different rA2ΔM2-2 isolates (Fig. 4B).

rA2ΔM2-2 protein synthesis. Since the putative M2-2 protein has not been identified in RSV-infected cells previously, it was necessary to demonstrate that the M2-2 protein is indeed encoded by RSV and produced in infected cells. We produced

TABLE 1. Levels of replication of rA2ΔM2-2 and rA2 in various cell lines[a]

Cell line	Host	Tissue origin	Titer (log_{10} Pfu/ml)	
			rA2	rA2ΔM2-2
Vero	Monkey	Kidney	6.1	6.1
HEp-2	Human	Larynx	6.2	4.3
MDBK	Bovine	Kidney	6.1	5.5
MRC-5	Human	Lung	5.5	3.0
HeLa	Human	Cervix	6.6	4.5
LLC-MK2	Monkey	Kidney	6.7	6.1

[a] Cells were infected with rA2 or rA2ΔM2-2 at an MOI of 0.2; at 72 h postinfection, the culture supernatants were harvested, and the levels of virus replication were determined by a plaque assay on Vero cells. Each virus titer was averaged from two experiments with two independent isolates for both viruses.

a polyclonal antiserum against the M2-2 fusion protein expressed in a bacterial expression system. Immunoprecipitation of rA2-infected Vero cell lysates with anti–M2-2 protein antibody produced a protein band of approximately 10 kDa, which is the predicted size for the M2-2 polypeptide. This polypeptide was not detected in rA2ΔM2-2-infected cells (Fig. 5A), confirming that the M2-2 protein is a product produced by RSV and that its expression was ablated from rA2ΔM2-2. The overall polypeptide pattern of rA2ΔM2-2 was indistinguishable from that of rA2. However, it was noted by immunoprecipitation that slightly higher levels of the P and SH proteins were produced in rA2ΔM2-2-infected Vero cells. Nevertheless, as noted by Western blotting analysis, comparable amounts of the SH protein were produced in cells infected with rA2ΔM2-2 or rA2 (Fig. 5B).

Western blotting was used to determine the rate and accumulation of protein synthesis by rA2ΔM2-2 in both Vero and HEp-2 cell lines. HEp-2 or Vero cells were infected with rA2ΔM2-2 or rA2; at various times postinfection, the infected cells were harvested, and the polypeptides were separated on a 17.5% polyacrylamide gel containing 4 M urea. The proteins were transferred to a nylon membrane and probed with polyclonal antisera against the three accessory proteins: M2-1, NS1 and SH. Protein expression levels for all three viral proteins were very similar for rA2ΔM2-2 and rA2 in both HEp-2 and Vero cells (Fig. 5B). Synthesis of the NS1 protein was detected at 10 h postinfection, slightly earlier than that of the M2-1 and SH proteins because the NS1 protein is the most abundant protein in infected cells due to its 3′ proximal location. Similar protein synthesis rates and levels were also observed for both rA2ΔM2-2 and rA2 when the membrane was probed with a polyclonal antiserum against RSV (data not shown). Comparable levels of the M2-1 protein were detected for both viruses, indicating that deletion of the M2-2 open reading frame did not affect the level of the M2-1 protein, which is translated by the same M2 mRNA.

Replication of rA2ΔM2-2 in mice and cotton rats. To evaluate the levels of attenuation and immunogenicity of rA2ΔM2-2, the replication of rA2ΔM2-2 in the upper and lower respiratory tracts of mice and cotton rats was examined. Mice in groups of six were inoculated with 10^6 PFU of rA2ΔM2-2 or rA2 intranasally. Animals were sacrificed at 4 days postinoculation; their nasal turbinates and lung tissues were harvested and homogenized, and levels of virus replication in these tissues were determined by a plaque assay. Geometric mean titers of virus replication and standard errors obtained from two experiments are shown in Table 2. rA2ΔM2-2 exhibited at least a 2.0-log unit reduction of replication in both nasal turbinates and lungs of infected mice. rA2ΔM2-2 replication was detected only in 1 or 2 of 12 infected mice. The replication was limited; only a few plaques were observed at a 10^{-1} dilution of the tissue homogenates. A high level of rA2 replication was detected in both the upper and the lower respiratory tracts of mice. A similar degree of attenuation of rA2ΔM2-2 was also observed in cotton rats. Despite its restricted replication in mice, rA2ΔM2-2 induced significant resistance to challenge with wild-type RSV A2 (Table 2). When mice previously inoculated with rA2ΔM2-2 or rA2 were inoculated intranasally with 10^6 PFU of wild-type A2 virus, no wild-type A2 virus replication was detected in the upper and lower respiratory tracts. Therefore, rA2ΔM2-2 was fully protective against wild-type A2 virus challenge.

The immunogenicity of rA2ΔM2-2 was also examined. Mice in groups of six were infected with rA2ΔM2-2 or rA2; 3 weeks later, serum samples were collected. The serum neutralization titer was determined by a 60% plaque reduction assay. The neutralization titer obtained from rA2ΔM2-2-infected mice was comparable to that of rA2; mice infected with both viruses had a 60% plaque reduction titer at mean dilutions of 1:32 to 1:64, whereas the prebleed serum had an RSV neutralization titer of 1:4. Since the detectable neutralization titer was low, as was also reported previously (17), we thus tested the RSV-specific antibody by immunostaining of RSV-infected cells. The serum obtained from rA2ΔM2-2-infected mice immunostained RSV plaques at dilutions similar to that of rA2, confirming that the RSV-specific antibody was produced in rA2ΔM2-2-infected mice at a level similar to that of rA2.

DISCUSSION

In this study, we reported the construction of a recombinant RSV that lacked the majority of the M2-2 open reading frame (rA2ΔM2-2). The recovery of rA2ΔM2-2 was confirmed by RT-PCR, sequencing, Northern blot hybridization, and immunoprecipitation analyses. A recombinant RSV that lacked M2-2 protein expression was viable in cell cultures, but it replicated poorly in several host cell lines and rodent hosts. The cell-type-dependent replication of rA2ΔM2-2 suggested that a host factor(s) might be involved in RSV replication in the absence of the M2-2 protein.

RSV encodes five unique proteins: NS1, NS2, SH, M2-1, and M2-2. It has been reported that the NS2 and SH genes are dispensable for RSV replication in vitro (3, 28). Our experiments demonstrated that the M2-2 gene is also not essential for RSV replication in cell cultures. Therefore, the M2-2 gene is the third gene that has been reported to be dispensable for RSV replication. It is very interesting that RSV has evolved to encode at least three nonessential genes in its genome. These accessory genes must therefore provide certain auxiliary functions for virus replication in hosts. Deletion of the RSV SH gene did not appear to affect virus replication in vitro (3), a result very similar to what was reported for recombinant SV5 lacking the SH gene (16). On the contrary, slightly better replication of the SH knockout RSV (rA2ΔSH) in certain cell lines has been observed. Although it is not attenuated in the lower respiratory tracts of mice (3), rA2ΔSH is attenuated in the lower respiratory tracts of chimpanzees (31). Removal of the NS2 gene impairs virus growth for both human RSV and bovine RSV (2, 28). The NS2 knockout RSV (rA2ΔNS2), in which tandem stop codons were introduced, reverted rapidly to restore NS2 protein expression (28). When inoculated into chimpanzees intranasally and intratracheally, rA2ΔNS2 is slightly attenuated in the upper respiratory tract but highly attenuated in the lower respiratory tract (31). These results

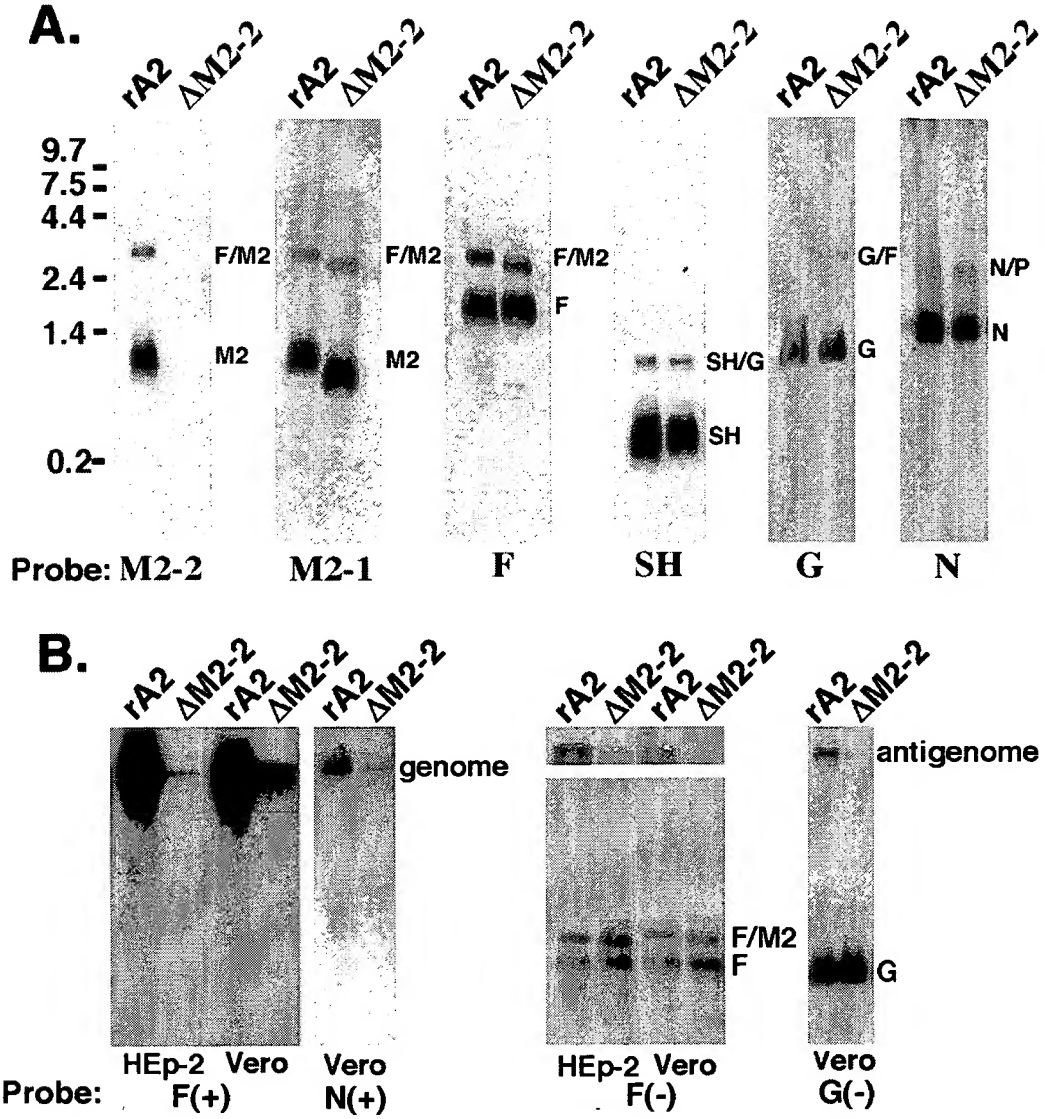

FIG. 4. Viral RNA expression by rA2ΔM2-2 and rA2. (A) Total RNA was extracted from rA2- or rA2ΔM2-2-infected Vero cells (MOI, 1.0) at 48 h postinfection, separated by electrophoresis on 1.2% agarose–2.2 M formaldehyde gels, and transferred to nylon membranes. Each blot was hybridized with a digoxigenin-labeled riboprobe specific for the M2-2, M2-1, F, SH, G, or N gene. The sizes of the RNA markers are indicated on the left. (B) HEp-2 and Vero cells were infected with rA2 or rA2ΔM2-2 for 24 h, and total cellular RNA was extracted. An RNA Northern blot was hybridized with a ^{32}P-labeled riboprobe specific for the negative-sense F gene to detect viral genomic RNA in both HEp-2 and Vero cells or an N gene probe to detect viral genomic RNA in Vero cells only. A ^{32}P-labeled riboprobe specific for the positive-sense F gene was used to detect viral antigenomic RNA and F mRNA in HEp-2 and Vero cells, and a G gene probe was used to detect antigenomic RNA and G mRNA in Vero cells only. The top panel of the Northern blot on the right (F gene probe) was taken from the top portion of the gel shown in the lower panel and was exposed for 1 week to show the antigenome. The lower panel of that Northern blot was exposed for 3 h to show the F mRNA. The genome, antigenome, F mRNA, and dicistronic F-M2 RNA are indicated.

indicate that the NS2 protein plays an important role in full virus replication capacity. The data presented in this study indicated that the M2-2 protein, although not essential for RSV viability, is an accessory factor that is able to substantially support virus growth in vitro and in vivo. Further studies are needed to understand the mechanisms by which the M2-2 protein facilitates virus growth in different cell types and in animal hosts.

The M2-2 protein has been identified as a strong inhibitor to RSV minigenome replication in vitro (9). The effect of inhibition of RNA synthesis by the M2-2 protein is reminiscent of the inhibitory transcription function of the M protein of vesicular stomatitis virus (5, 24). If this inhibitory effect is exerted late in infection, decreased RNA synthesis may be beneficial for the virus in restricting excess cytopathogenicity that may abort further progeny production and in rendering nucleocapsids quiescent prior to budding. Indeed, we have observed that cells infected with rA2ΔM2-2 exhibited a cytopathogenic effect earlier and had larger syncytia than cells infected with wild-type virus. However, down-regulation instead of up-regulation of RNA genome replication was observed in cells infected with rA2ΔM2-2. Much reduced amounts of antigenomic and genomic RNAs were detected in rA2ΔM2-2-infected cells. Northern blot hybridization data indicated that the amount of

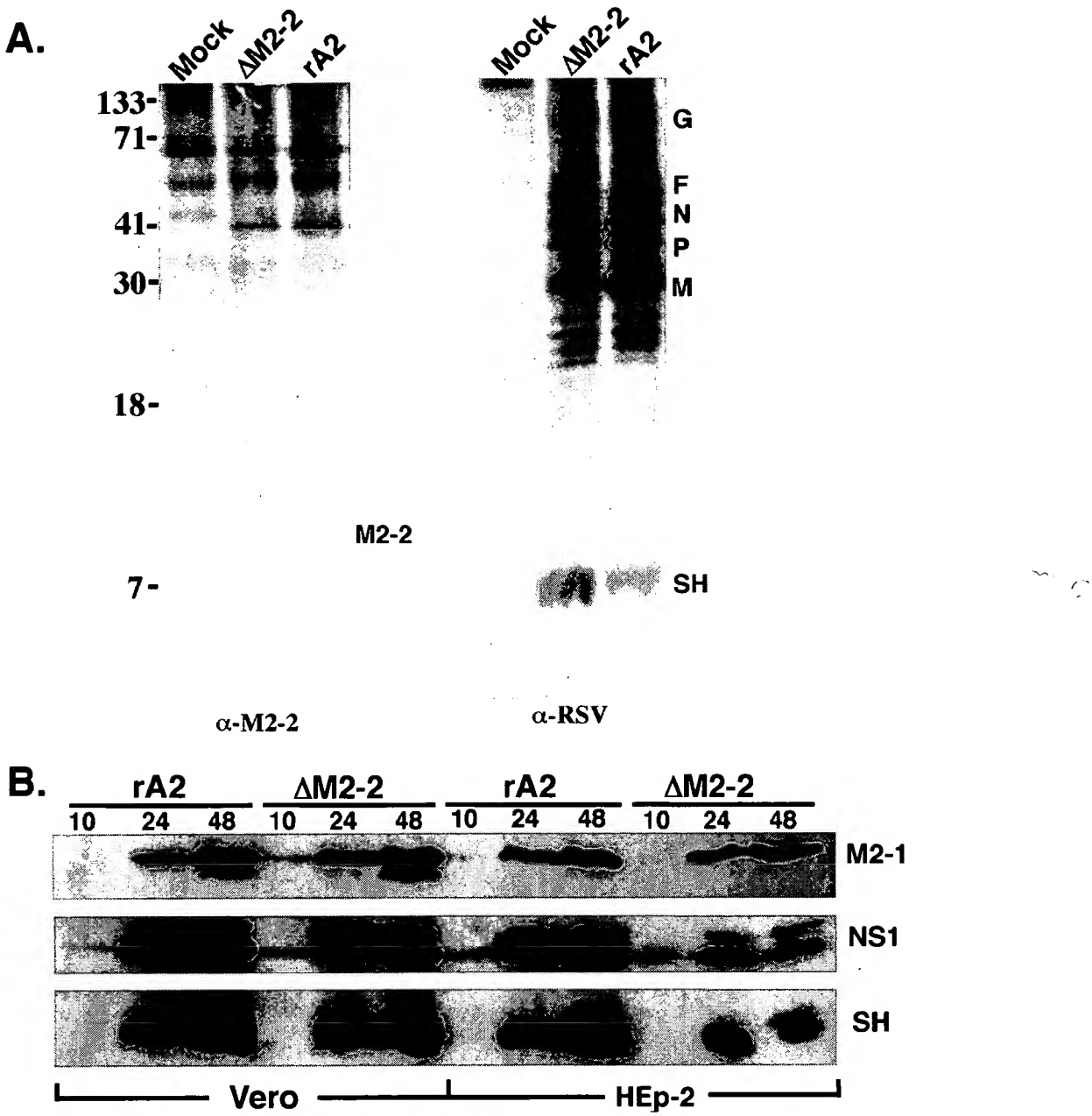

FIG. 5. Viral protein expression in rA2ΔM2-2- and rA2-infected cells. (A). Mock-infected or rA2ΔM2-2- and rA2-infected Vero cells (MOI, 1.0) were metabolically labeled with ^{35}S-promix (100 μCi/ml) between 14 and 18 h postinfection. Cell lysates were prepared for immunoprecipitation with goat polyclonal anti-RSV or rabbit polyclonal anti–M2-2 antiserum. Immunoprecipitated polypeptides were separated on a 17.5% polyacrylamide gel containing 4 M urea and processed for autoradiography. The position of each viral protein is indicated on the right, and the molecular weight size markers (in thousands) are indicated on the left. (B) Time course of RSV protein expression by rA2 and rA2ΔM2-2. HEp-2 and Vero cells were infected with rA2 or rA2ΔM2-2 at an MOI of 1.0. At 10, 24, or 48 h postinfection, total infected cellular polypeptides were separated on a 17.5% polyacrylamide gel containing 4 M urea. Proteins were transferred to a nylon membrane, and the blot was probed with polyclonal antisera against the M2-1, NS1, or SH protein.

mRNA production in rA2ΔM2-2-infected cells was comparable to that in cells infected with wild-type RSV. Viral proteins expressed by rA2ΔM2-2 were also comparable to those synthesized by wild-type RSV. The data obtained in experiments reported here did not provide any evidence that the M2-2 protein inhibited RSV RNA transcription and replication. The possibility that the M2-2 protein could affect mRNA stability in infected cells has not been examined. It is possible that the M2-2 protein is involved in the switch from RNA transcription to replication and thus results in reduced antigenomic and genomic RNA synthesis in cells infected with rA2ΔM2-2.

The HEp-2 cell line is fully permissive to wild-type RSV replication. However, replication of rA2ΔM2-2 in this cell line was reduced; rA2ΔM2-2 formed very small plaques and replicated to a low titer in HEp-2 cells. To delineate the M2-2 protein function that is required for efficient RSV replication

TABLE 2. Replication of rA2ΔM2-2 and rA2 in mice and protection against wild-type RSV A2 challenge

Immunizing virus	Virus replication[a] (mean $\log_{10}$ PFU/g of tissue ± SE) in:		RSV A2 replication after challenge[b] (mean $\log_{10}$ PFU/g of tissue ± SE) in:	
	Nasal turbinates	Lungs	Nasal turbinates	Lungs
rA2	3.72 ± 0.33	4.0 ± 0.13	<1.4	<1.4
rA2ΔM2-2	<1.4	<1.4	<1.4	<1.4
None (control)	<1.4	<1.4	3.53 ± 0.17	4.10 ± 0.13

[a] Groups of 12 BALB/c mice were immunized intranasally with 10^6 PFU of the indicated virus on day 0. The level of infected virus in the indicated tissues was determined by a plaque assay at day 4, and the mean $\log_{10}$ titer ± standard error per gram of tissue was determined.

[b] Groups of 6 BALB/c mice were challenged intranasally with 10^6 PFU of RSV A2 on day 21 and sacrificed 4 days later. The replication of wild-type RSV A2 in tissues was determined by a plaque assay, and the mean $\log_{10}$ titer ± standard error per gram of tissue was determined.

in HEp-2 cells, we compared RNA and protein syntheses of rA2ΔM2-2 and rA2 in both Vero and HEp-2 cells. Surprisingly, the amounts of RNAs and proteins expressed from rA2ΔM2-2-infected HEp-2 cells were comparable to those expressed from rA2-infected cells. The genomic and antigenomic RNA synthesis of rA2ΔM2-2 in HEp-2 cells is similar to that in Vero cells. Previously, it was reported that the addition of a small amount of the M2-2 protein increased the packaging of the minigenome in vitro (29). It is likely that the poor replication of rA2ΔM2-2 in HEp-2 cells is due to a defect at a later stage of virus replication, possibly during the virus assembly process. rA2ΔM2-2 formed very large syncytia in infected Vero cells. Preliminary data indicated that rA2ΔM2-2 is more fusogenic (data not shown) in a cytoplasmic content mixing experiment (16). How the lack of the M2-2 protein affected RSV fusion activity remains to be investigated.

Impaired virus replication and reduced virus pathogenicity due to the loss of virus accessory proteins have been reported for Sendai virus and measles virus. The C protein of Sendai virus inhibits viral mRNA synthesis and amplification of the Sendai virus minigenome in a promoter-specific manner (4, 27). However, up-regulation instead of down-regulation of transcription, translation, and genome replication was seen in Sendai virus that lacked the C protein. The virus lacking the C protein is highly attenuated in the natural murine host (22). The C protein of measles virus is dispensable for virus replication in Vero cells (25) but is required for efficient measles virus replication in human peripheral blood cells (12). The V protein of both Sendai virus and measles virus is also associated with virus pathogenicity (21, 30). As was observed when accessory genes were deleted from these other paramyxoviruses, we found that deletion of the M2-2 open reading frame rendered RSV attenuated in the upper and lower respiratory tracts of mice and cotton rats. Since other RSV proteins are targets of immunity (6), the absence of the M2-2 protein in a vaccine virus would not compromise the immunogenicity of RSV. rA2ΔM2-2 resembled rA2 in its ability to induce RSV-specific antibodies with neutralizing function and to protect mice against the replication of wild-type challenge virus in both the upper and lower respiratory tracts. Virus with M2-2 gene deletion is easily distinguishable from wild-type virus and genetically stable, making rA2ΔM2-2 a potential candidate vaccine for human use.

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Evaluation of recombinant respiratory syncytial virus gene deletion mutants in African green monkeys for their potential as live attenuated vaccine candidates

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Abstract

Towards the goal of developing live attenuated respiratory syncytial virus (RSV) vaccines to prevent severe respiratory tract infections caused by respiratory syncytial virus, recombinant RSV containing a deletion of single or multiple NS1, NS2, SH and M2-2 genes have been generated. In this study, recombinants, rA2ΔM2-2, rA2ΔNS2, rA2ΔNS1NS2, rA2ΔSHNS2, rA2ΔM2-2NS2 were evaluated in African green monkeys (AGMs) for their infectivity, immunogenicity and protection against wild type (wt) RSV challenge. Replication of rA2ΔNS2 and rA2ΔSHNS2 was not attenuated in either the upper or the lower respiratory tracts of AGMs. On the other hands, rA2ΔNS1NS2 was over-attenuated; it did not replicate in the respiratory tracts of the infected monkeys and did not provide sufficient protection against wild type RSV challenge. rA2ΔM2-2NS2 was slightly more attenuated than rA2ΔM2-2 and provided partial protection against wt RSV challenge. rA2ΔM2-2, and possibly rA2ΔM2-2NS2, exhibited the attenuated but protective phenotypes in the monkeys that could be further evaluated as potential live attenuated RSV vaccine candidates in the clinical studies.

Keywords: Respiratory syncytial virus (RSV); Immunogenicity; African green monkeys

1. Introduction

Respiratory syncytial virus (RSV) is the leading cause of severe viral bronchiolitis and pneumonia world-wide and accounts for approximately 100,000 hospitalizations and 4500 deaths in infants and young children in the Unites States each year [1,2]. RSV is also an important cause of severe respiratory illness in elderly [3–5] and immunocompromised populations [6,7]. To date, no effective licensed vaccine is available to prevent diseases associated with RSV infection.

Development of previous investigational RSV vaccines has been hampered by several different problems. Administration of formalin-inactivated vaccine in the 1960s resulted in enhanced RSV disease [8,9], subunit vaccines have exhibited poor immunogenicity [10,11], and live attenuated cold-passaged, temperature-sensitive (*cpts*) strains analyzed to date were inadequately attenuated in very young children [12,13]. A live attenuated RSV vaccine administered intranasally, however, is expected to mimic the mucosal, and systemic humoral and cellular immune responses elicited by natural infection without causing disease. In addition, live attenuated RSV does not potentiate disease caused by subsequent RSV infection [14,15], which provides a critical safety advantage over the inactivated vaccines [13]. The major challenges of developing a safe, effective, live attenuated RSV vaccine are achieving the appropriate modulation of virus attenuation, genetic stability of the attenuated vaccine strain and maintaining its ability to protect against both subgroup A and B RSV infection.

Reverse genetics has been applied to the field of RSV vaccine development to both evaluate and construct potential vaccine candidates. The attenuating mutations present in several *cpts* vaccine candidates have been identified by systematically introducing these changes individually and in combination into a full length RSV cDNA and evaluating the biological properties of the resulting recombinant virus [16,17]. Attenuating mutations from different *cpts* strains have been combined to produce recombinant vaccine candidates that are more attenuated in animal models than either parent strain. Novel recombinant attenuated RSV strains have also been produced by mutagenesis of essential internal

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genes such as L [18], P [19,20] and M2-1 in the A2 strain [21]. In addition, deletions of nonessential genes provide additional means to generate attenuated RSV. Deletion of NS1, NS2, SH or M2-2 alone or in combination from A2 strain have varying degrees of impact on viral replication in vitro [22–26] and in rodent and nonhuman primate models [25,27–29]. The phenotypes of these vaccines are expected to be genetically stable due to the nature of the lesion introduced into the genome. In this report, the attenuation and immunogenicity properties of several mutants with deletions in these nonessential genes of the RSV A2 strain were evaluated in African green monkeys (AGMs) to determine their potential as RSV vaccine candidates.

2. Materials and methods

2.1. Viruses and cells

Recombinant RSVs that contain deletions of M2-2 (rA2ΔM2-2), NS2 (rA2ΔNS2), SH and NS2 (rA2-ΔSHNS2), NS1 and NS2 (rA2ΔNS1NS2) or M2-2 and NS2 (rA2ΔM2-2NS2) were obtained by transfection of infectious RSV cDNA bearing the engineered gene deletions as described previously [24,25,30]. Each virus was plaque purified three times and amplified in Vero cells.

2.2. Infection of African green monkeys

Infection of RSV gene deletion mutants in African green monkeys (*Chlorocebus aethipos*) was conducted in two separate experiments. AGMs, average age of 4.4 years, were imported from St. Kitts of the Caribbean Sea and housed individually. The animal studies were approved by the Institutional Animal Care and Use Committee of Tulane National Primate Research Center and carried out in accordance with animal experimentation guidelines.

Animals were grouped as three or four AGMs per group and the control group was not vaccinated. Each monkey was inoculated with a dose of $10^{5.5}$ pfu of virus in a 0.9 ml of inoculum intranasally and an equal amount of virus was inoculated through the intratracheal route. After inoculation, nasopharyngeal (NP) swabs were collected daily from each monkey for 12 days using Telazol anesthesia. At each collection, two NP samples were collected by gently rubbing two to three regions of the oropharynx and placed in tubes containing 1.0 ml of Opti-MEM containing 1× SPG (0.2 M sucrose, 3.8 mM KH_2PO_4, 7.2 mM K_2PO_4 and 5.4 mM monosodium glutamate) and 0.1% gelatin. Bronchoalveolar lavage (BAL) samples were collected on days 3, 5, 7 and 10 post-infection [31]. For this procedure, 15 ml of sterile PBS was infused into the lung and 4–10 ml of fluid aspirated immediately using a sterile French catheter and syringe, and the samples were stabilized by addition of 0.2 volumes of a 5× concentrate of SPG and 0.5% gelatin. The NP and BAL samples were immediately frozen at −80 °C. On day 28 post-infection, serum samples were obtained and animals were boosted with the same virus at a dose of $10^{5.5}$ pfu by the intranasal and intratracheal routes. Virus shedding was examined in the NP and BAL samples on days 3, 5, 7, and 10 after the boosting immunization. On day 56, serum samples were collected from each immunized monkey and the animals were challenged with wild type (wt) A2 RSV by inoculating at both the intranasal and intratracheal sites with a dose of $10^{5.5}$ pfu in a total volume of 1.8 ml. The NP and BAL samples were collected on days 3, 5, 7 and 10 after the challenge infection. Fourteen days after wild type virus challenge, serum samples were collected to measure the levels of serum anti-RSV neutralizing antibody. The amount of virus shedding was quantitated by plaque assay on Vero cells. The detection limit of the plaque assay was 0.7 $\log_{10}$ pfu/ml and the undetectable virus titer was thus assigned as 0.35 $\log_{10}$ pfu/ml. The statistical significance in virus shedding between the vaccinated and control groups was determined by the Wilcox two-sample test.

2.3. Measurement of serum anti-RSV neutralizing antibody

Serum samples were collected prior to RSV infection, 28 days (post-immune serum), 58 days (post-boosting serum) and 72 days (post-challenge serum) after initial infection. The levels of anti-RSV neutralizing antibody were determined by microneutralization assay using rA2LacZ as neutralizing virus as described previously [27]. Briefly, monkey serum was treated at 56 °C for 30 min and two-fold serially diluted. Following incubation with 100 pfu of rA2LacZ at 4 °C for 2 h in a 96-well plate in the presence of 5% guinea pig complement (Invitrogen, Carlsbad, CA), Vero cells (50,000) were added to each well and the plates were incubated at 35 °C for 3 days. The cells were lysed with detergent and the lysate was incubated with 0.75 mM CPRG (chlorophenol red β-D-galactopyranoside, Roche Molecular Biochemicals, Indianapolis, IN) followed by measurement of the OD550 by spectrophotometry (Molecular Device, Sunnyvale, CA). The mean anti-RSV neutralizing antibody titer was defined as the reciprocal $\log_2$ of the highest antibody dilution that resulted in a 70% reduction in OD550 in comparison to unneutralized virus infection controls. The detection limit of the microneutralization assay is 3.0 $\log_2$ and a titer of <3.0 $\log_2$ was assigned for those that had no anti-RSV neutralizing antibody detected.

3. Results

3.1. Virus shedding

RSV mutants with deletions in one or more nonessential genes were evaluated in two separate experiments in AGMs to assess their levels of replication in the upper and

Table 1
Replication of RSV gene deletion mutants in the upper and lower respiratory tracts of African green monkeys

Virus[a]	No. of animals	Virus shedding (days)	Mean peak virus titer (log_{10}/ml ± S.E.)[b]	
			NP swab	Bronchoalveolar lavage
rA2	4	8–10	3.34 ± 0.23	3.36 ± 0.34
rA2ΔM2-2	3	1–5	0.94 ± 0.32[c]	1.42 ± 0.63[c]
rA2ΔNS2	3	5–8	2.98 ± 0.43	3.34 ± 0.26
rA2ΔSHNS2	4	7–9	3.63 ± 0.06	3.53 ± 0.41
rA2ΔNS1NS2	3	0	0.35[c]	0.35[c]
rA2ΔM2-2NS2	4	0–4	0.35[c]	0.72 ± 0.21[c]

[a] African green monkeys were administered with 5.5 log_{10} pfu of virus intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal lavage samples were collected on days 3, 5, 7 and 10.

[b] Virus titers were determined in the nasopharyngeal swab and bronchoalveolar lavage by plaque assay on Vero cells and shown here are peak virus titers that were detected between days 5–7. A titer of 0.35 log_{10} pfu/ml was assigned for those that did not shed virus.

[c] $P < 0.05$ (Wilcox two-sample test) compared with rA2-infected animals.

lower respiratory tracts, immunogenicity and ability to protect against wt RSV challenge. Recombinants, rA2ΔM2-2 and rA2ΔM2-2NS2 were used in study A and the remaining deletion mutants, rA2ΔNS2, rA2ΔSHNS2, rA2ΔNS1NS2 were employed in study B. Wt rA2 RSV was included in both experiments and replicated to comparable levels; thus the results of the two experiments are tabulated and discussed together. Replication of each RSV deletion mutant in comparison to wt rA2 in the upper and lower respiratory tracts of AGM is summarized in Table 1. Virus was shed from the nasopharynx of rA2-infected animals for 8–10 days with peak titers detected from days 5 to 7. The virus shedding from the nasopharynx of animals inoculated with the deletion mutants varied from 0 day (rA2ΔNS1NS2) to 7–9 days (rA2ΔSHNS2). Each gene deletion mutant exhibited varying level of replication in the upper and lower respiratory tracts of AGMs. The replication of rA2ΔM2-2 was reduced by 2.4 log_{10} and 1.9 log_{10} in the upper and lower respiratory tracts, respectively, compared to rA2. In contrast, rA2ΔNS2 replicated in both the upper and lower respiratory tracts of the AGMs to a level similar to that of wt rA2. The additional removal of the SH gene from rA2ΔNS2 did not attenuate this virus any further; rA2ΔSHNS2 had a peak titer (3.6 log_{10} pfu/ml in NP and 3.5 log_{10} pfu/ml in BAL) similar to that of wt rA2 (3.3 log_{10} pfu/ml and 3.4 log_{10} pfu/ml, respectively). In contrast, deletion of the NS1 gene from rA2ΔNS2 greatly decreased viral replication in the respiratory tracts of AGMs; virus shedding was not detected in either the upper or the lower respiratory tracts of AGMs infected with rA2ΔNS1NS2. Similarly, rA2ΔM2-2NS2 did not replicate in the upper respiratory tract and only a very low level of virus (0.7 log_{10} pfu/ml) was recovered from the lower respiratory tract of AGMs. Virus shedding was not detected after the boosting immunization in any of the virus groups (data not shown).

3.2. Immunogenicity and protection

Each RSV gene deletion mutant was further evaluated for its immunogenicity and protection against wt A2 RSV challenge (Table 2). In general, there was good correlation between viral replication following the first immunization of the animal and immunogenicity. The level of serum anti-RSV neutralizing antibody after one and two doses of

Table 2
Evaluation of recombinant RSV for their levels of immunogenicity and efficacy against wild type challenge virus in African green monkeys

Virus[a]	Neutralizing Ab titer (mean reciprocal log_2)[b]				Virus peak titer (mean log_{10} pfu/ml ± S.E.)[c]	
	Day 0	Day 28	Day 56	Day 72	Nasopharyngeal swab	Tracheal lavage
rA2	<3.0	8.8	9.0	12.2	0.35[d]	0.35[d]
rA2ΔM2-2	<3.0	6.6	9.7	10.5	0.91 ± 0.56[d]	0.35[d]
rA2ΔNS2	<3.0	8.8	11.0	12.0	0.35[d]	0.35[d]
rA2ΔSHNS2	<3.0	8.0	10.8	12.0	0.35[d]	0.35[d]
rA2ΔNS1NS2	<3.0	3.8	4.4	10.6	3.13 ± 0.68	1.56 ± 0.62[d]
rA2ΔM2-2NS2	<3.0	6.1	8.2	10.9	2.76 ± 0.90	0.69 ± 0.35[d]
Unvaccinated	<3.0	ND[e]	ND	8.4	3.96 ± 0.45	3.70 ± 0.26

[a] African green monkeys were administered with 5.5 log_{10} pfu of virus intranasally and intratracheally and on days 0 and 28. On day 56, monkeys were challenged with wt A2 at a dose of 5.5 log_{10} pfu intranasally and intratracheally.

[b] Serum anti-RSV neutralizing antibody were determined by microneutralization assay.

[c] Nasopharyngeal swab samples and tracheal lavage samples were collected on days 3, 5, 7 and 10 after challenge. Challenge virus titers were determined by plaque assay on Vero cells. Only the peak titers are shown. A titer of 0.35 log_{10} pfu/ml was assigned for those that did not shed virus.

[d] $P < 0.05$ (Wilcox two-sample test) compared with the unvaccinated group.

[e] ND: not determined.

vaccination and after wt A2 challenge virus infection was examined by the microneutralization assay [32]. An antibody response following primary vaccination was examined 2 weeks after infection (Table 2). Consistent with their protective ability, rA2ΔNS2 and rA2ΔM2-2NS2 were similar to wt rA2 in their ability to induce a serum anti-RSV neutralizing antibody response. The level of anti-RSV neutralizing antibody induced by rA2ΔM2-2 and rA2ΔM2-2NS2, however, was four to five-fold lower compared to rA2. However, this antibody response was augmented by the booster immunization and the level of neutralizing antibody elicited by rA2ΔM2-2 and rA2ΔM2-2NS2 boosting infection was similar or approaching that induced by rA2. The titer of neutralizing antibody stimulated by immunization with rA2ΔNS1NS2 was low, only slightly higher than that of the pre-immunization serum samples, which is consistent with its inability to replicate in the respiratory tracts of AGMs. Two weeks after challenge virus infection, anti-RSV neutralizing antibody levels were augmented for all the vaccinated animal groups, at least four-fold higher than the placebo animals that had not previously been immunized with RSV.

The challenge virus did not replication in either the upper or the lower respiratory tract of AGMs immunized with rA2, rA2ΔNS2 or rA2ΔSHNS2, and thus these strains provided complete protection. In animals immunized with rA2ΔM2-2, the challenge virus was detected in the upper respiratory tract at a level of approximate 1000-fold reduced compared to the unvaccinated control animals, no virus shedding was detected in the lower respiratory tract. rA2ΔM2-2NS2 provided less protection against wt challenge than rA2ΔM2-2. Animals immunized with rA2ΔM2-2NS2 had viral titers in the lower respiratory tract reduced by approximate 1000-fold compared to the unvaccinated control animals; however the impact on the level of challenge virus replication in the upper respiratory tract was more limited (about 12-fold lower than unvaccinated controls). Although replication of rA2ΔNS1NS2 was below the limit of detection in AGMs, this immunization still provided partial protection against wt challenge virus replication in the lower respiratory tract compared to the unvaccinated animals.

4. Discussion

Several animal models have been developed to evaluate RSV vaccine candidates for their attenuation, immunogenicity and protection against wild type RSV challenge. Animals susceptible to RSV infection include: nonhuman primates, cotton rats, mice, guinea pigs, ferrets and hamsters [33]. Recombinant RSVs with one or more accessory genes deleted have been evaluated in small animal models, including mice and cotton rats [24,25]. However, the replication defect of most of these deletion mutants in the upper and lower respiratory tracts of cotton rats [25] has made it difficult to select the most appropriate strain for clinical studies. rA2ΔNS2 and rA2ΔSHNS2 have been shown to be attenuated in cotton rats; virus replication was reduced by 75 to 100-fold in the lungs of these animals [25]. However, neither rA2ΔNS2 nor rA2ΔSHNS2 were attenuated in the upper and lower respiratory tracts of AGMs. Virus shedding was detected in rA2ΔNS1NS2-infected cotton rats [25] but not in AGMs that are more susceptible to RSV infection. These data indicated that nonhuman primate models are valuable for evaluation of RSV vaccine candidates.

Chimpanzee is the only experimental animal that approaches the human in permissiveness to RSV replication and disease [13,34]. This model has been used to evaluate attenuation and immunogenicity of RSV recombinants with alterations in the nonessential genes [28,29]. However, the expense and availability of chimpanzees have made it difficult to assess a large number of candidates in parallel in this model. AGM is less susceptible to RSV infection than chimpanzee, but it is more closely related to humans than the rodent models [31]. Recently, we have used AGMs to evaluate subgroup A and B RSV infection and showed that this animal model could serve as an alternative to chimpanzees for evaluating RSV vaccine candidates for their attenuation, immunogencity and protection against wild type RSV infection [27]. In this present study, several selected RSV mutants with deletions of nonessential genes were further evaluated in AGMs and our data indicated that rA2ΔM2-2, and possibly rA2ΔM2-2NS2, are promising vaccine candidates.

Previously, rA2ΔM2-2 was shown to be attenuated in both chimpanzees and AGMs [27,29]. rA2ΔM2-2 provided partial protection against wt challenge in AGMs after administration of a single dose [27]. Here, we show that administration of two doses of rA2ΔM2-2 provided better protection against wt RSV infection than observed in the previously published study. Complete protection from wt challenge virus replication was demonstrated in the lower respiratory tract and a 2.8 $\log_{10}$ reduction of the challenge virus titer in the upper respiratory tract of AGM was observed following vaccination with two doses of rA2ΔM2-2. These data indicate that multiple dosing will be a consideration for administration of live attenuated RSV vaccines. Additional removal of the NS2 gene from rA2ΔM2-2 (rA2ΔM2-2NS2) slightly increased virus attenuation without resulting in a significant reduction in its antibody response. This virus could potentially serve as an alternative vaccine candidate should the reactogenicity of rA2ΔM2-2 be greater than desired in infants.

A similarly constructed rA2ΔNS2 has been shown to be slightly attenuated in the upper respiratory tracts and greatly attenuated in the lower respiratory tracts of chimpanzees [28]. In addition, rA2ΔSH is attenuated in the lower respiratory tract of chimpanzees but not attenuated in the upper respiratory tract [28]. The data obtained from the present study demonstrated that recombinant RSVs bearing a deletion of the NS2 gene (rA2ΔNS2 and rA2ΔSHNS2) were not attenuated in either the upper or lower respiratory tract of AGMs.

It remains to be determined whether this discrepancy between the AGMs and chimpanzees studies reflected different susceptibilities of different animal hosts to these viruses or other explanations such as group sizes or sampling errors. How these results from either study relate to human remains to be investigated.

The data obtained from the in vitro and in vivo studies indicated that each RSV accessory protein played a different role in virus pathogenesis. Our studies indicated that the NS1 and M2-2 played a much more important role in RSV pathogenesis than NS2 and SH. The NS1 protein has been shown to be a potent inhibitor of viral RNA transcription and replication in vitro and the NS2 protein might have a similar inhibitory function [35]. The NS1 and NS2 proteins of bovine [36] and human RSV [37] have been shown to function as interferon antagonist in cultured cells. rA2ΔNS1 was not evaluated in ACMs but was evaluated in chimpanzees [29]. Replication of rA2ΔNS1 was significantly reduced in both the upper and lower respiratory tracts of the infected chimpanzees. Therefore, the attenuated phenotype of rA2ΔNS1NS2 may be conferred solely by the NS1 deletion. However, if both proteins are involved in interferon-antagonizing function, it is possible that deletion of both NS1 and NS2 genes acted in concert to produce a greater effect on virus replication in vivo. Evidence that deletion of NS2 contributes to attenuation of RSV is clear from the incremental attenuation of rA2ΔM2-2/NS2 compared to rA2ΔM2-2. Thus, it is possible that removal of the NS2 gene also incrementally attenuates a virus with a deletion in the NS1 gene.

The M2-2 protein is a strong inhibitor for viral RNA transcription and replication in a minigenome system [38]; the M2-2 protein has been shown to regulate RNA transcription and replication to facilitate virus morphogenesis [24,26]. The attenuated property of rA2ΔM2-2 did not appear to greatly impact its immunogenicity, and two doses of immunization induced a level of anti-RSV neutralizing antibody that provided an effective protection against wt challenge virus infection. These data supported the previous notion that rA2ΔM2-2 could he a very promising vaccine candidate for further clinical evaluation.

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Evaluation of Two Live, Cold-Passaged, Temperature-Sensitive Respiratory Syncytial Virus Vaccines in Chimpanzees and in Human Adults, Infants, and Children

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Two live-attenuated, cold-passaged *(cp)*, temperature-sensitive *(ts)* candidate vaccines, designated *cpts*530/1009 and *cpts*248/955, were attenuated, genetically stable, and immunogenic in chimpanzees and were highly attenuated for human adults. In respiratory syncytial virus (RSV)–seropositive children, *cpts*530/1009 was more restricted in replication than *cpts*248/955. In seronegative children, 10^4 pfu of *cpts*248/955 was insufficiently attenuated, and a high titer of vaccine virus was shed (mean peak titer, $10^{4.4}$ pfu/mL), whereas 10^4 pfu of *cpts*530/1009 was relatively attenuated and restricted in replication (mean peak titer, $10^{2.0}$ pfu/mL). At a dose of 10^5 pfu, *cpts*530/1009 was immunogenic in seronegative children (geometric mean titer of RSV neutralizing antibodies, 1:724). Transmission of either vaccine to seronegative placebo recipients occurred at a frequency of 20%–25%. Of importance, vaccine viruses recovered from chimpanzees and humans were *ts*. In contrast to previous studies, this study indicates that live attenuated RSV vaccines that are immunogenic and phenotypically stable can be developed. Additional studies are being conducted to identify a live RSV vaccine that is slightly more attenuated and less transmissible than *cpts*530/1009.

Respiratory syncytial virus (RSV) is the leading cause of viral respiratory illness in infants and children throughout the world (reviewed in [1]) and is an important cause of severe respiratory illness in the elderly [2] and in immunocompromised patients [3]. In the United States, RSV infections account for ~90,000 hospitalizations of children each year [4].

The importance of RSV as a respiratory pathogen makes RSV vaccine development a priority [5]. Since an effective vaccine will need to provide protective immunity against RSV-associated lower respiratory tract illness (LRI) in young infants, RSV immunization will need to be initiated in the first month of life. It may be difficult to immunize this population effectively for several reasons. Young infants may respond poorly to an RSV vaccine because of immunologic immaturity and because maternally derived antibody may interfere with the immune response to the vaccine [1, 6–8]. Infants will also need to be immunized with a vaccine that protects against the antigenically divergent RSV subgroups A and B. Finally, it is likely that infants will need to be immunized several times to achieve a satisfactory level of immunity since even a single infection with wild type (wt) RSV does not completely protect against subsequent RSV-associated LRI [9, 10].

Efforts to produce a safe and effective RSV vaccine have been underway for >30 years. Early attempts yielded a formalin-inactivated vaccine that produced enhanced disease in some immunized RSV-naive infants when they were naturally infected with wt RSV during the subsequent RSV season [11, 12]. More recently, an RSV fusion (F) subunit vaccine has been produced that may prove useful in the elderly [13] but is unlikely to be administered to young infants. Adenovirus and vaccinia virus recombinants containing the RSV F and attachment (G) glycoproteins have also been developed but were not sufficiently immunogenic in chimpanzees to warrant clinical evaluation [14–16].

Live RSV vaccines might provide the best alternative for immunizing young infants. A live vaccine would mimic natural infection, induce a balanced cellular and humoral immune response, and be unlikely to produce enhanced disease [6]. In addition, live virus vaccines can replicate at mucosal surfaces even in the presence of passively acquired antibodies [7, 8, 17]. Previous live RSV candidate vaccines were either over- or underattenuated in young children [18–21], and those that were temperature-sensitive *(ts)* did not retain this phenotype during replication in vivo [22].

Recently, a series of live attenuated candidate vaccines was derived by chemical mutagenesis of an incompletely attenuated cold-passaged RSV mutant (*cp*RSV [23–25]). The parent virus,

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Guidelines for human experimentation of the Joint Committee for Clinical Investigation of the Johns Hopkins University School of Medicine and the Institutional Review Board of Vanderbilt University Medical Center were followed in the conduct of this study.

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*cp*RSV, a non-*ts* strain, contained mutations that restricted viral replication in adults and RSV-seropositive children but was insufficiently attenuated for RSV-seronegative children [19]. *cp*RSV was subjected to two rounds of chemical mutagenesis, and *ts* mutant derivatives (referred to as *cpts*) were generated [23–25]. It was hoped that the non-*ts* attenuating mutations present in the *cp*RSV parent virus would act in concert with the *ts* mutations to yield attenuated, genetically stable vaccines. Several *cpts* RSV mutants were subsequently shown to be restricted in replication in BALB/c mice and in chimpanzees [23, 24]. The *ts* phenotype of two of these mutants was more stable than that present in the prototype live attenuated RSV vaccine virus, *ts*-1 [23]. Here we describe the preclinical and phase I evaluation of RSV *cpts*248/955 and *cpts*530/1009 in chimpanzees and adults and in RSV-seropositive and -seronegative infants and children. For purposes of comparison, wt RSV strain A2 and *cp*RSV were also evaluated in adult volunteers and chimpanzees.

Materials and Methods

Viruses. The isolation and characterization of wt RSV strain A2, of *cp*RSV, and of the *ts* mutants 248/955 (shutoff temperature, 37°C) and 530/1009 (shutoff temperature, 36°C) have been described [24–27]. Each of these *ts* mutants was derived independently from *cp*RSV by serial mutagenesis with 5-fluorouracil in Vero cell monolayer cultures. The *ts* mutants were biologically cloned by three plaque-to-plaque passages and amplified by four passages to prepare the vaccine pools. The wt RSV strain A2 (designated lot RSV M6) was prepared in MRC-5 cell monolayer cultures, and each of the progeny strains (*cp*RSV, lot A-11; *cpts*248/955, lot A-10; and *cpts*530/1009, lot A-16) were prepared in qualified Vero cell monolayer cultures. The *cp*RSV lot A-11 differed from the previously evaluated *cp*RSV [27] in that it was biologically cloned by three plaque-to-plaque passages in MRC-5 cell monolayer cultures. This cloned preparation of *cp*RSV served as the immediate parent for the *cpts* derivative viruses. Virus suspensions for clinical trials were produced and found to be free of adventitious agents by Louis Potash (Dyncorp/PRI, Bethesda, MD). The titers of the wt RSV A2 strain and of *cp*RSV, *cpts*248/955, and *cpts*530/1009 were $10^{3.9}$, $10^{5.8}$, $10^{5.9}$, and $10^{5.5}$ pfu/mL, respectively. When necessary, the virus suspensions were diluted in L-15 medium (BioWhittaker, Walkersville, MD) immediately prior to use.

Studies in chimpanzees. Young male or female chimpanzees *(Pan troglodytes)* weighing 8.8–10.4 kg were pair-housed in large glass isolator suites and maintained as described previously [28]. The animals given *cpts*248/955 were on loan from the University of Texas MD Anderson Cancer Center (Bastrop). These chimpanzees lacked detectable serum neutralizing antibodies to RSV A2 (titer <1:10). Four seronegative chimpanzees were inoculated with *cpts*248/955 by both the intranasal and intratracheal routes with a dose of 10^4 pfu in a 1-mL inoculum at each site. Data from similar animals that received wt RSV strain A2, *cp*RSV, and *cpts*530/1009 were described previously [23, 25] and are presented here for the purpose of comparison. The comparability of the studies was insured by the use of identical protocols and challenge virus suspensions. In addition, inoculation, sampling, and clinical scoring procedures were performed by the same individuals in each study. Following inoculation of virus, nasopharyngeal swab specimens were collected while animals were under ketamine anesthesia for quantitation of the amount of virus shed on days 1–10, 13, 16, and 20, and tracheal lavage specimens were collected on days 2, 4, 6, 8, 10, 13, 16, and 20, as described previously [15]. Virus present in the respiratory tract secretions was quantified by plaque titration at 32°C, 39°C, and 40°C on HEp-2 cells as previously described. The amount of rhinorrhea was estimated daily and assigned a score of 0 to 4 by an experienced observer (0 = none, 1 = trace, 2 = mild, 3 = moderate, 4 = severe [29]). One month after immunization, animals were challenged with wt RSV A2 virus as previously described [25].

Clinical studies of adults. The RSV A2 wt virus, *cp*RSV, *cpts*248/955, and *cpts*530/1009 were each evaluated in open-label, nonrandomized trials in healthy adults 18–45 years of age who were not in contact with immunosuppressed individuals or infants <1 year of age. Ninety-nine adults participated in these studies: 44 received the RSV wt A2 virus, 20 each received the *cp*RSV or the *cpts*248/955 viruses, and 15 received *cpts*530/1009. The health of the adult volunteers was assessed as previously described [29].

The wt RSV A2 virus was evaluated in the Johns Hopkins University Center for Immunization Research (CIR) isolation unit. Volunteers were given $10^{3.9}$ pfu of RSV A2 wt virus in 1 mL intranasally. Nasal washes to quantitate virus shedding were performed daily, once prior to inoculation and for 10 days after. Volunteers were examined each day, and their temperatures and vital signs were recorded every 6 h during the 13-day isolation period. Vaccine strains were evaluated in outpatient studies at the CIR. Volunteers who received 10^5 pfu of *cp*RSV, *cpts*248/955, or *cpts*530/1009 intranasally were examined on the day of inoculation and on days 4–8 following inoculation. On each study day (0–10), volunteers recorded their own oral temperatures twice and reported any respiratory or febrile illness to the study nurse. All subjects who reported illness were examined by a study investigator.

Clinical studies of children. After *cpts*248/955 and *cpts*530/1009 were shown to be well tolerated in adults, these strains were evaluated in randomized, double-blind, placebo-controlled phase I trials in infants and children 6–60 months of age at the CIR and at the Vanderbilt University Vaccine Center (VVC). Ninety children were enrolled in these phase I safety and immunogenicity studies: 40 participated in studies of *cpts*248/955 and 50 in studies of *cpts*530/1009. Children were eligible to participate in these studies if they were healthy and if all other household members and day care contacts were ≥1 year of age. Prior to enrollment, children were screened for level of RSV serum neutralizing antibody by a complement-enhanced, 60% plaque-reduction neutralization assay [30]; those with titers >1:40 were considered RSV-seropositive. Both vaccines were initially tested at a dose of 10^5 pfu in seropositive children and tested subsequently at a dose of 10^4 pfu (*cpts*248/955 strain) or at 10^4 or 10^5 pfu (*cpts*530/1009 strain) in seronegative children. Each subject received 0.5 mL of vaccine or placebo intranasally. In the pediatric studies, the ratio of vaccinees to placebo recipients was ~2:1. Seropositive study participants were examined 2 days before inoculation and for 9 days after. Seronegative study participants were examined 2 days

before and on days 1–9, 11, 14, 16, 18, 21, and 23 after inoculation; interval symptom histories were obtained from parents on days when the children were not examined. Children were observed for 1–2 h at the CIR and for 6–10 h at the VVC in a playroom setting on each study day. Respiratory and febrile illnesses were defined as fever (rectal temperature, ≥38.1°C), upper respiratory tract illness (URI; rhinorrhea or pharyngitis for ≥2 days), LRI (persistent wheezing or pneumonia), and cough (on ≥2 consecutive days) [31].

To assess the long-term safety of the *cpts* 248/955 and 530/1009 candidate vaccines, seronegative infants and children enrolled in these trials were followed through the subsequent RSV season. A group of RSV-seronegative children who did not receive vaccine or placebo were recruited as additional control subjects for this phase of the study. Children who participated in surveillance were monitored throughout the RSV season for fever and respiratory illnesses (as defined above). Nasal washes from ill children were tested for RSV by culture and by EIA (Testpack; Abbott Laboratories, Abbott Park, IL).

Isolation, quantitation, identification, and phenotypic characterization of virus. Nasal wash specimens for virus isolation were obtained on each day of observation from all subjects who participated in these studies. Fresh undiluted nasal wash specimens were titered by plaque assay on HEp-2 cell monolayer cultures maintained under a semisolid overlay at 32°C as previously described, and results were expressed as $\log_{10}$ pfu/mL [25]. Nasal wash samples were also inoculated into tubes containing Vero and HEp-2 cell culture monolayers. Virus isolates from these tubes were identified as RSV using an indirect IFA (Bartels Microscan; Baxter Healthcare, Bellevue, WA). For purposes of calculation, samples in which virus was not detected or did not produce plaques were assigned an infectivity titer of $10^{0.6}$ pfu/mL.

Phenotypic characterization of virus isolates. To assess the stability of the *ts* phenotype, fresh nasal wash specimens from subjects who participated in trials of *cpts*248/955 and *cpts*530/1009 were titered at 32°C and 40°C, and a more extensive analysis of efficiency of plaque formation at 32°C, 38°C, 39°C, and 40°C was determined subsequently using frozen aliquots of nasal wash specimens as previously described [23–25].

Immunologic assays. Sera and nasal wash specimens for measurement of RSV-specific antibodies were obtained from adults and RSV-seropositive children before and 4 weeks after inoculation and from RSV-seronegative children before and 8 weeks after inoculation. Sera were tested for antibodies to RSV by plaque reduction neutralization assay and for IgG antibodies to RSV F and G glycoproteins by end-point titration in an ELISA using immunoaffinity-purified F and G glycoproteins from RSV A2 infected cell lysates [32, 33].

Nasal wash samples were also tested for the presence of IgA antibody to purified RSV F and G glycoproteins by ELISA. Each ELISA nasal wash anti-RSV F or G IgA titer was corrected to a total IgA concentration of 100 mg/mL as measured by a radial immunodiffusion assay (Binding Site, San Diego) as previously described [29].

Data analysis. Laboratory evidence of infection with an RSV wt or vaccine strain was defined as isolation of RSV, a ≥4-fold rise in serum RSV neutralizing antibody titer, and/or a ≥4-fold rise in serum IgG antibody titer to purified RSV F and/or G glycoproteins. In several persons, isolated serum responses to either RSV F or G glycoprotein were detected. In these instances, the ELISAs for both F and G glycoproteins were repeated and, if the response was confirmed, these individuals were considered to have been infected with the candidate vaccine virus. Isolated nasal wash antibody responses to RSV F or G glycoprotein were not considered definitive evidence of infection with vaccine virus.

RSV antibody titers were expressed as reciprocal mean $\log_2$. The Mann-Whitney *U* test (two-tailed) was used to compare mean titers between groups. Rates of illness among vaccinees and placebo recipients were compared by two-tailed Fisher's exact test.

Results

Response of RSV-seronegative chimpanzees to wt RSV A2, cpRSV, cpts248/955, or cpts530/1009. Of the viruses studied, *cpts*530/1009 was the most restricted in replication in the upper and lower respiratory tracts of seronegative chimpanzees (table 1). Of importance, the replication of both *cpts*248/955 and 530/1009 was highly restricted in the lower respiratory tract, suggesting that it would be safe to evaluate these vaccines in clinical trials. RSV *cpts*248/955 recovered from nasopharyngeal specimens (n = 30) and tracheal lavage specimens (n = 5) failed to produce plaques at 40°C, indicating that the *cpts*248/955 candidate vaccine, like the previously evaluated *cpts*530/1009 candidate vaccine, retained the *ts* phenotype after replication in seronegative chimpanzees [25]. The 4 chimpanzees that received the *cpts*248/955 candidate vaccine developed a moderate titer of neutralizing antibody (geometric mean titer, 1:478) and, like the chimpanzees that received the *cpts*530/1009 candidate vaccine, were completely resistant to challenge with wt RSV A2 virus [25].

Response of adult volunteers to wt RSV A2, cpRSV, cpts248/955, or cpts530/1009. *cp*RSV, *cpts*248/955, and *cpts*530/1009 replicated less well than wt virus in healthy adults (table 2). The 3 mutant viruses were less infectious than wt virus and were shed less frequently in these subjects (P = .01, .003, and .001 for *cp*RSV, *cpts*248/955, and *cpts*530/1009, respectively; Fisher's exact test). Respiratory, febrile, or systemic illnesses also occurred less often in recipients of these attenuated strains than in recipients of wt RSV A2 (P = .006, <.001, and <.001 for *cp*RSV, *cpts*248/955, and *cpts*530/1009, respectively; Fisher's exact test). Serum or nasal wash antibody responses to wt virus or vaccine were observed in about one-third of the study participants; the rate of response did not differ significantly between any of these groups (table 3). The attenuation of the *cpts*248/955 and 530/1009 candidate vaccines for healthy adults led us to evaluate these *cpts* viruses in RSV-seropositive children. The biologically cloned *cp*RSV was not evaluated in children because previous studies indicated that uncloned *cp*RSV was insufficiently attenuated in RSV-seronegative infants [19].

Response of RSV-seropositive children to cpts248/955 or cpts530/1009. The *cpts*248/955 and *cpts*530/1009 candidate vaccines were evaluated at a dose of 10^4 or 10^5 pfu in seroposi-

Table 1. Response of RSV-seronegative chimpanzees to intranasal and intratracheal infection with 10^4 pfu of wild type RSV A2, *cp*RSV, A2 *cpts*248/955, or *cpts*530/1009.

RSV administered	No. of chimpanzees	No. infected	Rhinorrhea score mean (SD)	Mean peak titer* (SD) of virus in	
				Nasopharynx	Trachea
Wild type	4	4	1.4 (0.9)	5.5 (0.4)	5.7 (0.3)
*cp*RSV	2	2	0.6 (0.1)	4.6 (0.5)	2.9 (0.1)
*cpts*248/955	4	4	0.9 (0.2)	4.6 (0.8)	1.6 (1.6)
*cpts*530/1009	4	4	0.5 (0.3)	3.6 (0.5)	1.0 (0.6)

NOTE. For purposes of calculation, titer of 0.7 pfu/mL was assigned to culture-negative samples.
* Virus titers are expressed as $\log_{10}$ pfu/mL.

tive children (table 2). *cpts*248/955 infected the majority of vaccinees at each dose tested, and children who received 10^5 pfu of this candidate vaccine shed virus in titers as high as $10^{4.7}$ pfu/mL (mean, $10^{2.7}$). In contrast, *cpts*530/1009 infected few vaccinees and was not recovered from any seropositive child, indicating that it was more attenuated than *cpts*248/955 for seropositive children. The absence of LRI in seropositive recipients of the *cpts*248/955 and 530/1009 candidate vaccines suggested that it was safe to continue the evaluation of these *cpts* viruses in RSV-seronegative children.

The local and systemic immune responses of seropositive children to each of these candidate vaccine viruses are shown in table 3. At the 10^5-pfu dose, a serum neutralizing or glycoprotein ELISA antibody response was observed in 62% of the vaccinees who received *cpts*248/955 and 31% of those who received *cpts*530/1009. Nasal antibody responses to either candidate vaccine were detected less frequently in these seropositive children. A single placebo recipient in the *cpts*248/955 vaccine study developed a 4-fold rise in serum antibody titer to the RSV F glycoprotein, which might have

Table 2. Clinical and virologic responses of adults and seropositive and seronegative children to wild type RSV A2, *cp*RSV, *cpts*248/955, *cpts*530/1009, or placebo.

Subjects	Virus given	Dose ($\log_{10}$ pfu)	No. of subjects	% infected	Virus isolation (nasal wash)			% with indicated illness					
					% shedding	Duration of shedding, mean (SD)	Peak titer, mean (SD) $\log_{10}$ pfu/mL	Fever	URI	LRI	Cough	Otitis media	Any RSV-like illness
Adults	Wild type	3.9	44	50	43*†‡	6.8 (2.8)	3.3 (1.5)	7	41	7	0	0	52§‖¶
	*cp*RSV	5.0	20	30	10*	2.7 (3.8)	1.7 (2.1)	5	10	0	0	5	15§
	248/955	5.0	20	10	5†	4.0 (4.0)	2.8 (2.2)	0	5	0	0	0	5‖
	530/1009	5.0	15	33	0‡	0	≤0.6	0	0	0	0	0	0¶
Children													
Seropositive	248/955	4.0	6	67	17	5.3 (9.1)	1.1 (0.9)	67	67	0	0	17	100
	530/1009	4.0	5	0	0	0	≤0.6	40	0	0	20	0	60
	248/955	5.0	13	62	38	5.1 (4.0)	2.7 (1.8)	15	7	0	0	0	23
	530/1009	5.0	13	31	0	0	≤0.6	15	0	0	0	0	15
	Placebo††	0.0	9	0	0	0	≤0.6	44	0	0	0	11	55
	Placebo‡‡	0.0	7	0	0	0	≤0.6	14	0	0	0	0	14
Seronegative	248/955	4.0	8	88	88	9.0 (1.9)	4.4 (1.0)	88	88	12	25	25	100
	530/1009	4.0	7	86	43	4.8 (5.7)	2.0 (1.5)	57	71	0	0	14	71
	530/1009	5.0	8	100	100	11.0 (1.7)	4.5 (1.8)	62	62	12	0	38	88
	Placebo††	0.0	4	25	25	23	3.7	75	100	0	25	0	100
	Placebo‡‡	0.0	10	20	10	8.0 (8.0)	1.2 (0.6)	40	60	10	0	20	80

NOTE. Six- to 60-month-old RSV-seropositive children and 6- to 36-month-old RSV-seronegative children were enrolled in these studies. For purposes of this study, seropositive children were those with RSV serum plaque-reduction neutralizing antibody titers >1:40. Infection was defined as described in text. URI, upper respiratory tract illness; LRI, lower respiratory tract illness. Duration of shedding is defined as last day on which vaccine virus was recovered.
* $P = .01$, † .003, ‡ .001; § .006; ‖ <.001; ¶ <.001.
†† = placebo recipients in studies of RSV A2 *cpts*248/955 virus.
‡‡ = placebo recipients in studies of RSV A2 *cpts*530/1009 virus.

Table 3. Immunologic responses of adults and RSV-seropositive and -seronegative children to wild type RSV A2, *cp*RSV, *cpts*248/955, *cpts*530/1009, or placebo.

Subjects, virus given	Dose (log_{10} pfu)	*n*	% infected	% with any serum antibody response	Mean RSV serum NA titer (SD)			Mean RSV serum ELISA titer (SD)						Mean nasal wash ELISA titer (SD)					
								F glycoprotein			G glycoprotein			F glycoprotein			G glycoprotein		
					Before	After	% with ≥4-fold rise	Before	After	% with ≥4-fold rise	Before	After	% with ≥4-fold rise	Before	After	% with ≥4-fold rise	Before	After	% with ≥4-fold rise
Adults																			
Wild type	3.9	44	50	39	NT	NT	—	10.8 (1.7)	10.9 (1.6)	16	10.4 (1.7)	10.9 (1.7)	30	4.6 (1.7)	5.5 (2.1)	36	4.6 (2.1)	6.0 (2.3)	36
*cp*RSV	5.0	20	30	30	10.0 (1.3)	9.9 (1.6)	0	10.4 (1.3)	10.4 (1.2)	15	9.7 (1.4)	10.1 (1.5)	20	4.7 (2.1)	5.7 (1.8)	15	4.4 (1.9)	5.2 (2.4)	30
248/955	5.0	20	10	10	10.0 (1.0)	9.9 (1.0)	5	10.6 (1.3)	10.6 (1.3)	5	10.1 (1.2)	9.9 (1.3)	0	4.5 (1.5)	4.2 (1.6)	5	5.0 (1.6)	5.2 (2.0)	10
530/1009	5.0	15	33	33	9.9 (1.1)	10.4 (1.0)	7	10.4 (1.4)	11.0 (1.4)	27	9.8 (1.9)	9.8 (1.4)	20	4.7 (2.1)	5.1 (1.7)	20	5.2 (2.4)	6.1 (2.1)	27
Children																			
Seropositive																			
248/955	4.0	6	67	67	8.9 (1.0)	10.3 (0.6)	33	9.0 (2.1)	10.0 (0.9)	50	7.0 (2.1)	7.0 (2.1)	0	3.2 (0.7)	5.1 (1.2)	17	2.4 (1.2)	4.3 (1.9)	17
530/1009	4.0	5	0	0	7.3 (2.3)	7.5 (2.4)	0	10.9 (0.8)	10.5 (1.0)	0	9.3 (2.8)	8.5 (2.0)	0	4.2 (1.3)	3.1 (1.1)	0	4.2 (0.7)	3.1 (0.3)	0
248/955	5.0	13	62	62	9.2 (1.3)	9.9 (1.0)	8	11.0 (1.7)	11.0 (2.1)	31	8.2 (1.2)	9.5 (1.5)	54	4.5 (2.5)	5.7 (2.3)	15	3.0 (2.0)	3.9 (2.0)	8
530/1009	5.0	13	31	31	10.0 (1.0)	10.4 (0.8)	15	10.1 (1.7)	10.5 (1.5)	31	7.5 (1.8)	7.9 (2.1)	31	4.6 (1.8)	4.6 (1.6)	8	3.0 (1.4)	2.7 (1.2)	0
Placebo*	0.0	9	11	11	9.1 (1.3)	8.9 (1.3)	0	11.2 (2.0)	10.7 (1.3)	11	8.7 (1.2)	8.3 (1.0)	0	3.6 (2.2)	5.1 (1.9)	0	2.6 (1.6)	3.8 (2.6)	11
Placebo†	0.0	7	0	0	9.1 (2.7)	9.0 (2.7)	0	9.6 (2.9)	9.0 (2.9)	0	7.0 (2.3)	6.4 (2.1)	0	5.2 (1.9)	4.7 (2.0)	0	2.9 (1.3)	2.9 (0.9)	0
Seronegative																			
248/955	4.0	8	88	88	≤4.6 (0.0)	9.6 (2.1)	88	6.6 (2.0)	9.1 (1.9)	75	6.6 (1.4)	8.3 (1.7)	62	3.8 (2.6)	3.9 (1.3)	25	3.8 (2.6)	4.2 (1.5)	25
530/1009	4.0	7	86	86	≤4.6 (0.0)	8.1 (2.7)	57	7.0 (2.2)	8.3 (1.5)	57	6.2 (1.8)	7.0 (1.4)	57	2.0 (2.0)	4.3 (1.6)	43	1.8 (1.8)	3.1 (2.3)	28
530/1009	5.0	8	100	100	≤4.6 (0.0)	9.5 (0.6)	100	7.6 (2.1)	8.8 (2.2)	88	7.8 (1.9)	10.3 (1.0)	75	4.9 (1.3)	4.6 (3.2)	12	5.1 (1.3)	5.6 (2.8)	25
Placebo*	0.0	4	25	25	≤4.6 (0.0)	5.8 (1.3)	25	5.3 (1.4)	5.8 (1.7)	25	5.8 (1.7)	5.8 (1.7)	25	2.7 (1.7)	1.8 (0.6)	0	2.7 (1.7)	2.1 (1.0)	25
Placebo†	0.0	10	20	20	≤4.6 (0.0)	5.3 (2.0)	10	6.7 (2.5)	6.3 (2.0)	20	6.9 (2.2)	6.6 (1.4)	20	2.3 (2.1)	3.6 (2.6)	10	2.0 (1.3)	2.6 (2.0)	0

NOTE. All titers are expressed as mean reciprocal log_2. Nasal wash antibody titers were corrected to 100 mg/mL total IgA. For purposes of calculation, nasal wash antibody titers ≤0 were assigned value of 0.1. NT, not tested; NA, neutralizing antibody.

* Placebo recipients in studies of RSV A2 *cpts*248/955 virus.

† Placebo recipients in studies of RSV A2 *cpts*530/1009 virus.

resulted from transmission of vaccine virus from an infected vaccinee.

Response of RSV-seronegative children to cpts248/955 or cpts530/1009 vaccines. At a dose of 10^4 pfu, *cpts*248/955 was infectious and immunogenic but not sufficiently attenuated for RSV-seronegative infants and children (table 2). Eighty-eight percent of seronegative infants and children shed vaccine virus (mean peak titer, $10^{4.4}$ pfu/mL). Respiratory or febrile illness or otitis media associated with shedding of *cpts*248/955 was observed in all infected children, 1 of whom also had a viral enanthem consistent with enterovirus infection. One child who received *cpts*248/955 had 3 days of LRI (wheezing) associated with viral shedding (figure 1). This child, who was treated as an outpatient, received nebulized bronchodilators and oral bronchodilators and steroids and recovered uneventfully. Because the *cpts*248/955 candidate vaccine retained the capacity to cause LRI, its clinical evaluation was terminated. This virus was also transmitted to a placebo recipient, who shed virus ($10^{3.7}$ pfu/mL) and had rhinorrhea and cough on days 21–23.

At a dose of 10^4 pfu, RSV *cpts*530/1009 was more attenuated than *cpts*248/955. The mean peak titer of virus shed by seronegative vaccinees was $10^{2.0}$ pfu/mL, which was less than that shed by seronegative recipients of the *cpts*248/955 candidate vaccine ($P = .01$, Mann-Whitney U test). LRI was not observed in children who received 10^4 pfu of the *cpts*530/1009 candidate vaccine. At a dose of 10^5 pfu, clinical evaluation of *cpts*530/1009 was complicated by simultaneous infection with adenovirus in 3 vaccinees and 1 placebo recipient. LRI was observed in 1 vaccinee and 1 placebo recipient, who both shed adenovirus and *cpts*530/1009, but was not observed in other study participants. The *cpts*530/1009 mutant was apparently transmitted to this placebo recipient, who shed $10^{1.7}$ pfu of vaccine virus on a single day (day 16) that did not coincide with her LRI (days 18–20). URI, low-grade fever, and otitis media occurred frequently but at approximately the same rate in vaccinees and placebo recipients (table 2).

The *cpts*248/955 and 530/1009 candidate vaccines were highly immunogenic in RSV-seronegative children (table 3). Rises in neutralizing or F or G antibody titers were detected in 88% of those who received 10^4 pfu of *cpts*248/955 and all of those who received 10^5 pfu of *cpts*530/1009. Nasal antibody responses were detected less frequently than serum antibody responses in these children, perhaps because of the insensitivity of the assay as compared with that of serologic assays.

A total of 23 seronegative vaccinees (8 of whom received *cpts*248/955 and 15 of whom received *cpts*530/1009) and 64 unvaccinated children (13 placebo recipients and 51 control

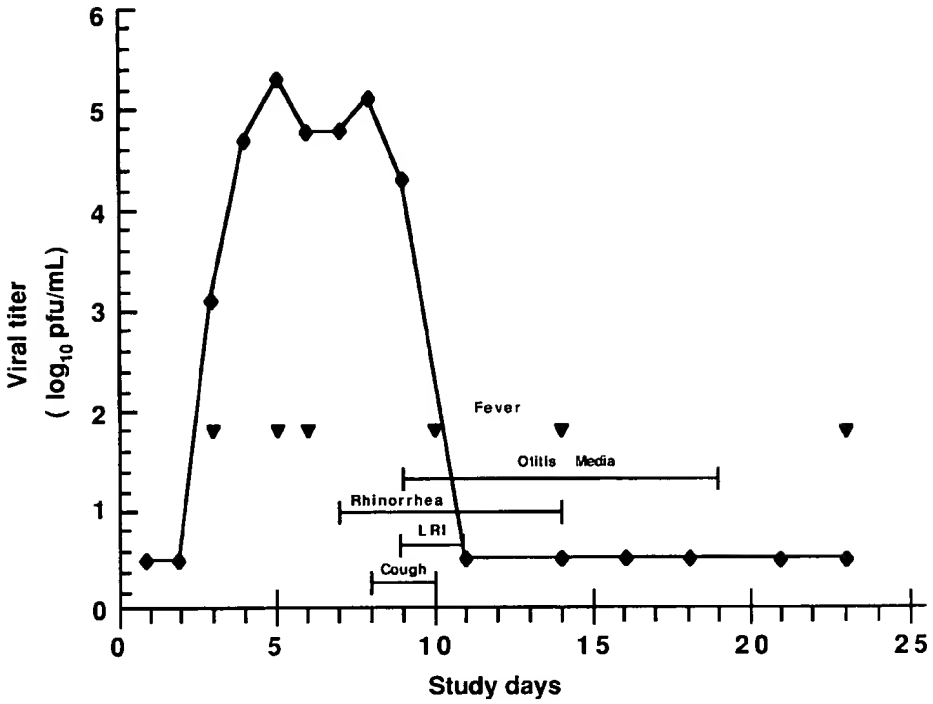

Figure 1. Response of seronegative vaccinee to 10^4 pfu of RSV A2 *cpts*248/955 vaccine.

subjects) participated in RSV surveillance. Twelve (52%) of the vaccinees and 31 (48%) of the unvaccinated subjects were infected with wt RSV during surveillance. Of the infected vaccinees, all had URI, 2 had fever, 2 had otitis media, and one had LRI. The single subject with LRI (wheezing and crackles on auscultation) was a recipient of *cpts*530/1009 who did not shed vaccine virus but had developed a neutralizing antibody response to RSV. Of the infected unvaccinated subjects, all had URI, 11 had fever, 8 had otitis media, and 3 had LRI (all 3 children wheezed; 1 also had crackles on auscultation). Thus, there was no evidence of enhanced disease when recipients of these live RSV candidate vaccines were infected with wt RSV.

Phenotypic stability of the cpts248/955 and 530/1009 vaccines. Despite a moderate to high level of replication in the upper respiratory tracts of RSV-seropositive and -seronegative children, the *cpts*248/955 candidate vaccine retained the *ts* phenotype: Each of 40 nasal wash specimens containing virus produced plaques at 32°C but not at 40°C. Similarly, virus present in each of 71 specimens from vaccinees infected with the *cpts*530/1009 candidate vaccine retained the *ts* phenotype. The efficiency of plaque formation of virus present in the nasal washes of 5 *cpts*530/1009 vaccinees (table 4) indicated that little drift in the level of temperature sensitivity occurred during viral replication in fully susceptible children over an interval of 9–14 days.

Discussion

The strategy of passaging virus at low temperature to yield mutants that replicate efficiently at suboptimal temperature but are restricted in replication at core human body temperature has been used successfully to generate cold-adapted *(ca) ts* vaccines against influenza virus and parainfluenza virus type 3 (reviewed in [34]), [35]. Earlier attempts to develop RSV vaccines that were either *cp* or *ts* were initially abandoned because of residual virulence for RSV-seronegative infants and because of the genetic instability of the RSV *ts*-1 mutant [1]. Recently, *cp*RSV was further mutagenized to generate a series of *cpts* vaccine candidates that were shown to be attenuated and genetically stable in mice and seronegative chimpanzees [15, 23–25]. The *cp*RSV evaluated in this study was a biologically cloned derivative of the *cp*RSV previously evaluated in the 1960s and 1970s. In the present study, we found that the cloned *cp*RSV, the immediate parent of the *cpts* vaccines, was attenuated in adults. This indicates that the biologically cloned *cp*RSV contains non-*ts* attenuating mutations that should be present in its *cpts* derivatives. Indeed, the *cpts* viruses sequenced to date contain the five nucleotide mutations [36] present in the biologically cloned *cp*RSV parent virus. Thus, one or more of these non-*ts* mutations attenuate *cp*RSV for chimpanzees and adults.

In this study, we also demonstrated that the *cp*RSV, *cpts*248/955, and *cpts*530/1009 viruses showed a progressive increase in attenuation for seronegative chimpanzees and adults. However, the *cpts*248/955 candidate vaccine was not sufficiently attenuated for fully susceptible (i.e., RSV-seronegative) children because it was shed in large quantities from the upper respiratory tract, temporally associated with rhinorrhea in 6 children and with LRI in 1 child, and transmitted to a placebo

Table 4. Characterization of the *ts* phenotype of virus recovered from 5 seronegative children who received 10^5 pfu of RSV A2 *cpts*530/1009 vaccine.

Assay no.	Vaccinee no. or virus	Study day	Titer of virus in nasal aspirate (log_{10} pfu/mL) at indicated temperature (°C)			
			32	38	39	40
1	26591	4	3.8	<0.7	<0.7	<0.7
		5	4.6	<0.7	<0.7	<0.7
		6	5.3	<0.7	<0.7	<0.7
		7	5.2	<0.7	<0.7	<0.7
		8	>6.5	3.6*	<0.7	<0.7
		9	4.5	1.1*	<0.7	<0.7
		11	4.6	<0.7	<0.7	<0.7
1	26878	6	4.7	<0.7	<0.7	<0.7
		7	5.5	<0.7	<0.7	<0.7
		8	4.5	<0.7	<0.7	<0.7
		9	3.6	<0.7	<0.7	<0.7
2	141	9	4.6	<0.7	<0.7	<0.7
		11	2.9	<0.7	<0.7	<0.7
		14	1.0	<0.7	<0.7	<0.7
3	589	3	1.3	<0.7	<0.7	<0.7
		4	3.0	<0.7	<0.7	<0.7
		5	4.1	<0.7	<0.7	<0.7
		6	4.7	<0.7	<0.7	<0.7
		7	4.5	2.0*	<0.7	<0.7
		8	4.6	<0.7	<0.7	<0.7
		9	5.1	<0.7	<0.7	<0.7
		10	3.7	<0.7	<0.7	<0.7
		11	3.0	<0.7	<0.7	<0.7
3	590	3	3.7	<0.7	<0.7	<0.7
		4	4.1	<0.7	<0.7	<0.7
		5	3.6	<0.7	<0.7	<0.7
		6	6.4	<0.7	<0.7	<0.7
		7	5.9	2.0*	<0.7	<0.7
		8	3.7	<0.7	<0.7	<0.7
		9	5.5	<0.7	<0.7	<0.7
		10	5.8	<0.7	<0.7	<0.7
		11	4.5	<0.7	<0.7	<0.7
1	*cpts*530/1009	—	5.9	3.5*	<0.7	<0.7
2	*cpts*530/1009	—	5.2	<0.7	<0.7	<0.7
3	*cpts*530/1009	—	5.9	<0.7	<0.7	<0.7
1	A2 wild type	—	>6.5	>6.5	>6.5	>6.2†
2	A2 wild type	—	>6.5	>6.5	>6.5	>6.5
3	A2 wild type	—	>6.5	>6.5	>6.5	>6.5

* Pinpoint plaque phenotype (<10% wild type plaque size at 32°C).
† Small plaque phenotype (<50% wild type plaque size at 32°C).

recipient. These studies suggest that RSV candidate vaccines, such as *cpts*248/955, that replicate to high titer in seropositive children may not be sufficiently attenuated for seronegative children. Evaluation of the *cpts*530/1009 candidate vaccine in seropositive children showed that this virus was more restricted in replication and more attenuated than *cpts*248/955. The clinical evaluation of *cpts*530/1009 in seronegative children was complicated by concurrent adenovirus infection in 4 subjects (and LRI in 2 subjects infected with both viruses); however, LRI was only observed in the children who shed adenovirus, which suggests that *cpts*530/1009 may be more attenuated than *cpts*248/955 in these susceptible children. Of importance, there was no evidence of disease enhancement when recipients of either of these live RSV candidate vaccines were infected with wt RSV.

The *cpts*248/955 vaccine was highly attenuated for chimpanzees and yet was able to cause LRI in seronegative children. This was an unexpected finding, and the reasons for this difference in response are not known. However, the relative order of attenuation of these viruses in chimpanzees (wt A2 virus being the most virulent, followed sequentially by *cp*RSV, *cpts*248/955, and *cpts*530/1009) was identical to that observed in our clinical studies, indicating that preclinical evaluation in chimpanzees provides valuable information about live RSV A vaccines destined for evaluation in humans.

In seronegative children, the *cpts*248/955 and 530/1009 candidate vaccines were highly infectious and immunogenic. The geometric mean titers of RSV neutralizing antibody achieved in recipients of 10^4 pfu of the 248/955 vaccine (1:776) and of 10^5 pfu of 530/1009 vaccine (1:724) were well above the level believed necessary to protect the lower respiratory tracts of susceptible infants [37]. It is hoped that similar levels of neutralizing antibodies might be induced in seronegative children with further attenuated *cpts* RSV vaccines, especially if more than one dose of vaccine is administered. These further attenuated *cpts* vaccines are, however, minimally infectious in adults and RSV-seropositive children, so it is likely that other vaccines will be needed to prevent serious RSV disease in the elderly and in RSV-seropositive children with chronic lung disease [2, 38].

The *cpts*248/955 and 530/1009 candidate vaccines were each recovered from a single seronegative placebo recipient. This is not surprising, since wt RSV spreads rapidly through susceptible populations [1, 9], and previous studies of the RSV *ts*-1 candidate vaccine showed that *ts* virus was recovered from a study nurse and a placebo recipient [18, 22]. In addition, some of the seronegative vaccinees in our studies shed virus in titers as high as $10^{5.0}$ pfu/mL, which would likely exceed the dose required to infect a susceptible contact. Of note, the vaccine virus recovered from placebo recipients retained the *ts* phenotype. It may be that a live attenuated RSV vaccine, like live poliovirus vaccines, will retain the ability to spread to contacts, and studies of future live RSV candidate vaccines will need to address this possibility.

The stability of the *ts* phenotype of the *cpts*248/955 and 530/1009 candidate vaccines was assessed by determining the efficiency of plaque formation of virus present in 40 nasal wash specimens of those who received the 248/955 candidate vaccine and 71 nasal wash specimens of those who received the 530/1009 candidate vaccine. None of the virus present in the nasal washes produced plaques at 40°C, indicating that the *ts* phenotype was maintained despite vigorous replication of these viruses in the upper respiratory tracts of RSV-seronegative subjects. This is the first evidence that live RSV candidate vaccines

can be produced that maintain the *ts* phenotype after replication in seronegative children because previous studies of the RSV *ts*-1 vaccine in chimpanzees and young children showed that ts^+ revertant virus could be recovered from nasal wash specimens [18, 29]. It is not known why the *cpts* live RSV candidate vaccines have a more stable phenotype than RSV *ts* candidate vaccines. It may be that the stability of the *ts* phenotype in the *cpts* vaccines is augmented by additional non-*ts* attenuating mutations already present in the *cp* parent virus, as has been observed with cold-adapted influenza virus vaccines [39]. These findings are encouraging and provide the basis for continued development and evaluation of *cpts* candidate vaccines.

In summary, we have shown that live *cpts* RSV candidate vaccines can be produced that are phenotypically stable following replication in RSV-seronegative children. A further attenuated derivative of *cp*RSV, *cpts*248/404, has been developed [25], and this vaccine may prove to be more restricted in replication and less transmissible than *cpts*530/1009. Evaluation of the *cpts*248/404 vaccine is in progress.

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MAJOR ARTICLE

Identification of a Recombinant Live Attenuated Respiratory Syncytial Virus Vaccine Candidate That Is Highly Attenuated in Infants

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(See the editorial commentary by Englund, on pages 1036–39.)

Background. Recombination technology can be used to create live attenuated respiratory syncytial virus (RSV) vaccines that contain combinations of known attenuating mutations.

Methods. Two live attenuated, recombinantly derived RSV vaccine candidates, rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH, were evaluated in 31 adults and in 95 children ≥6 months old. rA2cp248/404/1030ΔSH was subsequently evaluated in 44 infants 1–2 months old. These vaccine candidates share 4 attenuating genetic elements and differ only in a missense mutation (1030) in the polymerase gene.

Results. Both vaccines were highly attenuated in adults and RSV-seropositive children and were well tolerated and immunogenic in RSV-seronegative children. Compared with that of rA2cp248/404ΔSH, replication of rA2cp248/404/1030ΔSH was restricted in RSV-seronegative children (mean peak titer, $10^{4.3}$ vs. $10^{2.5}$ plaque-forming units [pfu]/mL), indicating that the 1030 mutation had a potent attenuating effect. Although rA2cp248/404/1030ΔSH was well tolerated in infants, only 44% of infants who received two $10^{5.3}$-pfu doses of vaccine had detectable antibody responses. However, replication after administration of the second dose was highly restricted, indicating that protective immunity was induced. At least 4 of 5 attenuating genetic elements were retained in recovered vaccine viruses.

Conclusions. rA2cp248/404/1030ΔSH is the first RSV vaccine candidate to be sufficiently attenuated in young infants. Additional studies are needed to determine whether rA2cp248/404/1030ΔSH can induce protective immunity against wild-type RSV.

Respiratory syncytial virus (RSV) is the most important cause of viral lower respiratory tract illness (LRI) in infants and children [1], but a vaccine is not available because of several obstacles, including the need to vaccinate early in life [2–4] and the history of immune-mediated enhancement of naturally occurring RSV disease among RSV-naive recipients of a formalin-inactivated RSV vaccine [5–7]. Enhanced RSV disease has never been observed after natural infection or administration of candidate live attenuated RSV vaccines [2, 8–10]. Live virus vaccines administered intranasally also afford better mucosal immunity than do inactivated virus vaccines administered parenterally [11, 12].

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For these reasons, live attenuated vaccines are being developed for RSV-naive populations.

Live attenuated RSV vaccines have been in development for several decades. A cold-passaged *(cp)*, non–temperature sensitive *(ts)* derivative of RSV, *cp*RSV, caused mild respiratory illness in young children [13]. Chemical mutagenesis of *cp*RSV produced several *ts* mutants [14–16] that were evaluated in clinical trials during the 1990s. *cpts*248/955 was insufficiently attenuated in RSV-seronegative children and older infants, precluding further evaluation in younger infants [9]. However, *cpts*248/404 was highly attenuated in RSV-seronegative children and was the first RSV vaccine to be administered to 1–2-month-old infants. Unfortunately, *cpts*248/404 caused nasal congestion in these infants, an unacceptable adverse effect in this population. The nasal congestion was temporally associated with vaccine virus shedding, and the mean peak titers shed were $10^{4.0}$ and $10^{4.9}$ pfu/mL at the 2 dose levels tested [2]. Although *cpts*248/404 was insufficiently attenuated in this target population, this study provided important information regarding (1) the level of attenuation necessary for infants, (2) the ability of live attenuated RSV to replicate and induce antibody responses in infants who have maternally derived RSV antibody, and (3) preliminary evidence of protection against illness associated with wild-type (wt) RSV infection [2].

Efforts were next made to develop a live RSV vaccine that was slightly more attenuated than *cpts*248/404 in RSV-naive infants and children. The mutations present in *cp*RSV and 6 *cpts* derivatives were identified by means of cDNA technology [17], permitting the generation of recombinant RSV (rRSV) vaccine candidates that contained new combinations of attenuating *cp* and *ts* point mutations [18–21] and deletions (Δ) of nonessential genes (e.g., the SH, NS1, NS2, and M2-2 genes) [22, 23]. On the basis of preclinical studies, 1 deletion mutation (ΔSH) and 1 *ts* mutation (1030) were selected for addition to *cpts*248/404, to generate rRSVs that might be more attenuated in humans. ΔSH attenuated wt RSV in mice [22] and chimpanzees. Although the levels of attenuation of 248/404ΔSH and *cpts*248/404 were similar in chimpanzees [23], ΔSH was chosen because studies in infants might show an additional attenuating effect, given that the young infant is a more permissive host for RSV than the chimpanzee [2]. The 1030 mutation was added to create rA2cp248/404/1030ΔSH, because rA2cp248/404/1030 was more *ts* in vitro and more attenuated in mice than was *cpts*248/404 [24].

rA2cp248/404ΔSH contains 4 independent attenuating genetic elements: *cp*, which is based on 5 missense mutations in the N and L proteins and the F glycoprotein that together confer the non-*ts* attenuation phenotype of *cp*RSV and that are considered to be a single attenuating genetic element [25]; *ts*248, a missense mutation in the L protein [16, 19]; *ts*404, a nucleotide substitution in the gene-start transcription signal of the M2 gene [20]; and ΔSH, complete deletion of the SH gene [20, 23, 25]. rA2cp248/404/1030ΔSH contains 5 independent attenuating genetic elements: those present in rA2cp248/404ΔSH and *ts*1030, another missense mutation in the L protein [24]. Here, we report the evaluation of the safety, immunogenicity, and phenotypic stability of rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH in adult and pediatric populations.

PARTICIPANTS, MATERIALS, AND METHODS

Vaccines. Construction, rescue, and biological cloning of infectious rA2 RSV strains were conducted at the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, MD); Wyeth-Ayerst Research (Gosport, United Kingdom); and Wyeth Vaccines (Pearl River, NY) [18, 24, 25]. rA2cp248/404ΔSH was rescued and biologically cloned by means of 3 successive plaque-to-plaque purifications and was amplified twice, all in HEp-2 cells. Virus seed was passaged once in Vero cells and was purified by means of 3 successive plaque-to-plaque isolations in Vero cells. The final virus clone was amplified by means of 1 passage in Vero cells and 1 passage in Vero cell microcarrier culture at 30°C.

rA2cp248/404/1030ΔSH was rescued in HEp-2 cells and biologically cloned by means of 2 additional terminal dilutions. The final virus clone was amplified by means of 2 passages in Vero cells followed by 1 passage in Vero cell microcarrier spinner culture. This material was used to prepare the vaccine in microcarrier culture at 30°C.

Viral suspensions for clinical trials were produced in Vero cells and were found to be free of adventitious agents by Wyeth Vaccines. The titers of rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH were $10^{7.0}$ pfu/mL and $10^{7.5}$ pfu/mL, respectively. To achieve the necessary titers, vaccines were diluted as described elsewhere [2]. Diluent was also used as placebo.

Study design. Both rRSV vaccines were evaluated in open-label trials in adults and in randomized, double-blind, placebo-controlled trials in RSV-seropositive and RSV-seronegative children (table 1). rA2cp248/404/1030ΔSH was also evaluated in infants. The studies were conducted between April and November over several years. One RSV-seronegative placebo recipient and 2 recipients of $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH were naturally infected with community-acquired wt RSV, as determined by sequence analysis of recovered virus. Their data were excluded from the analysis of safety and immunogenicity (tables 1 and 2) but were included in comparisons of phenotypic stability (table 3).

Eligible individuals were healthy and had no contact with immunosuppressed individuals or infants <6 months old [2, 9]. Each 0.5-mL dose of vaccine or placebo was intranasally administered. After inoculation, physical examinations were performed, and nasal wash specimens were obtained for viral culture

Table 1. Clinical and virologic responses of adults, children, and infants to rA2cp248/404ΔSH, rA2cp248/404/1030ΔSH, and placebo.

Participants, virus given	Dose, log_{10} pfu	No. of participants	Participants infected, %	Virus isolation, nasal wash			Participants with indicated illness, %						
				Participants who shed virus, %	Duration of shedding, mean (±SD), days	Peak titer, mean (±SD), log_{10} pfu/mL	Fever	URI	Nasal congestion	LRI	Cough	OM	Any respiratory or febrile illness
Adults													
rA2cp248/404ΔSH	5.0	15	13	13	5.5 (0.7)	1.6 (0.1)	0	7	NT	0	0	7	13
rA2cp248/404ΔSH	4.3	16	0	0	0	≤0.3	0	0	NT	0	0	0	0
Children													
RSV seropositive													
rA2cp248/404ΔSH	5.0	10	10	10	4.0	1.6	10	10	NT	0	0	10	20
rA2cp248/404/1030ΔSH	4.3	12	8	8	12.0	1.4	17	8	NT	0	8	0	25
rA2cp248/404/1030ΔSH[a]	5.3	13	8	8	8.0	2.4	38	38	NT	8[b]	23	8	69
Placebo[c]	...	5	0	0	0	≤0.3	0	60	NT	0	20	0	60
Placebo[d]	...	10	0	0	0	≤0.3	0	30	NT	0	30	20	40
RSV seronegative													
rA2cp248/404ΔSH	5.0	8	100	100	14.1 (2.7)	4.3 (1.1)	50	75	NT	0	25	25	88
rA2cp248/404/1030ΔSH	4.3	13	69	62	13.4 (8.8)	2.6 (1.3)	62	77	NT	0	31	23	92
rA2cp248/404/1030ΔSH	5.3	8	100	100	15.8 (7.2)	2.5 (0.7)	38	38	NT	0	25	13	50
Placebo[c]	...	3	33	0	0	≤0.3	33	33	NT	0	0	0	67
Placebo[d]	...	13	0	0	0	≤0.3	46	54	NT	8	23	0	69
Infants													
rA2cp248/404/1030ΔSH													
First dose	4.3	16	63	63	10.1 (2.7)	2.4 (1.3)	6	38	19	13[b]	38	0	69
Second dose	4.3	14	29	29	11.8 (4.6)	1.5 (0.8)	7	29	43	0	29	7	64
rA2cp248/404/1030ΔSH													
First dose	5.3	16	94	94	10.6 (3.7)	3.5 (0.8)	19	19	44	0	6	0	56
Second dose	5.3	16	44	44	12.7 (7.1)	1.3 (0.6)	13	19	25	0	25	13	31
Placebo													
First dose	...	12	0	0	0	≤0.3	8	8	8	0	8	0	17
Second dose	...	9	0	0	0	≤0.3	33	33	22	0	0	11	44

NOTE. Nasal congestion was assessed only in infants. Children were tested for levels of serum antibodies to respiratory syncytial virus (RSV) by 60% plaque reduction neutralization assay [26]. Those with titers >1:40 were considered to be RSV seropositive, and those with titers ≤1:40 were considered to be RSV seronegative [9]. Physical examinations were performed and nasal wash specimens were obtained on study days 0, 3–7, and 10 for adults and RSV-seropositive children and on study days 0, 3, 5, 7; day 8 or 9; day 10 or 11; and days 12, 14, 21, and 28 for RSV-seronegative children. For infants, physical examinations were performed and nasal wash specimens were obtained on study days 0; 4 or 5; 7, 8, or 9; 11 or 12; 16, 17, or 18; and 28. Interim history was obtained by telephone on the study days on which the participants were not evaluated. Studies were conducted at the Johns Hopkins University Center for Immunization Research (Baltimore, MD), the Saint Louis University Vaccine Evaluation Unit (St. Louis, MO), and the Vanderbilt Vaccine Clinic (Nashville, TN). Duration of shedding was defined as the time between inoculation and the last day on which vaccine virus was recovered. Participants were considered to be infected if at least 1 of the following criteria was met: vaccine virus was isolated, a ≥4-fold increase in neutralizing antibody titer occurred, or ≥4-fold increases in ≥2 of the ELISA titers (serum IgG to RSV F glycoprotein, serum IgG to RSV G glycoprotein, serum IgA to RSV F glycoprotein, and serum IgA to RSV G glycoprotein) occurred. LRI, lower respiratory tract illness; NT, not tested; OM, otitis media; URI, upper respiratory tract illness.

[a] Includes 3 children who were found to be RSV seronegative at the initial screening but were found to be RSV seropositive when serum specimens were retested with a neutralization assay that included complement.

[b] Three vaccine recipients had LRI; none shed vaccine virus, but other viruses—enterovirus (1 participant), rhinovirus (1 participant), and parainfluenza virus type 3 (1 participant)—were detected in nasal wash specimens by culture or polymerase chain reaction.

[c] Placebo recipients in studies of rA2cp248/404ΔSH vaccine.

[d] Placebo recipients in studies of rA2cp248/404/1030/ΔSH vaccine.

Table 2. Serum antibody responses of adults, children, and infants to rA2cp248/404ΔSH, rA2cp248/404/1030ΔSH, and placebo.

Participants, virus given	Dose, log_{10} pfu	Participants		RSV neutralizing antibody titer, mean (±SD)			ELISA IgG titer, mean (±SD)						ELISA IgA titer, mean (±SD)					
							F glycoprotein			G glycoprotein			F glycoprotein			G glycoprotein		
		Total	With any antibody response, %	Before	After	≥4-fold increase, %[a]	Before	After	≥4-fold increase, %[a]	Before	After	≥4-fold increase, %[a]	Before	After	≥4-fold increase, %[a]	Before	After	≥4-fold increase, %[a]
Adults																		
rA2cp248/404ΔSH	5.0	15	0	8.5 (1.0)	8.6 (1.1)	0	14.1 (0.8)	14.1 (0.8)	0	13.4 (1.3)	13.4 (1.1)	0	8.8 (1.5)	8.9 (1.5)	0	9.6 (2.0)	9.8 (2.1)	0
rA2cp248/404/1030ΔSH	4.3	16	6	7.9 (0.7)	7.9 (0.6)	0	14.3 (1.2)	14.2 (1.3)	0	13.0 (1.5)	13.1 (1.9)	6	8.8 (2.3)	9.0 (2.0)	0	8.8 (7.2)	8.6 (2.2)	0
Children																		
RSV seropositive																		
rA2cp248/404ΔSH	5.0	10	0	7.8 (1.7)	8.0 (1.9)	0	15.3 (0.9)	15.1 (1.4)	0	12.4 (0.9)	12.3 (1.2)	0	9.7 (1.6)	9.8 (1.6)	0	7.4 (1.5)	6.6 (1.8)	0
rA2cp248/404/1030ΔSH	4.3	12	8	8.6 (1.4)	8.6 (1.3)	0	15.1 (1.6)	15.0 (1.3)	0	12.2 (1.9)	12.0 (1.5)	8	8.0 (1.7)	8.0 (1.6)	0	4.8 (2.7)	4.7 (2.5)	0
rA2cp248/404/1030ΔSH	5.3	13	15	8.7 (1.2)	8.5 (1.3)	0	16.4 (2.3)	15.8 (1.8)	0	11.3 (3.9)	11.6 (4.1)	15	10.3 (2.4)	9.5 (2.5)	0	5.8 (3.1)	5.7 (3.3)	0
Placebo[b]	...	5	0	8.3 (1.0)	7.7 (1.9)	0	15.0 (1.0)	14.8 (1.0)	0	11.8 (2.3)	11.8 (2.6)	0	9.4 (1.9)	9.5 (1.7)	0	6.2 (1.3)	6.3 (1.0)	0
Placebo[c]	...	10	0	8.1 (1.5)	8.0 (1.3)	0	15.9 (2.1)	15.9 (1.9)	0	11.4 (3.9)	10.9 (3.7)	0	9.4 (2.2)	9.5 (2.0)	0	5.4 (2.9)	5.7 (2.9)	0
RSV seronegative																		
rA2cp248/404ΔSH	5.0	8	88	3.3 (0.0)	6.6 (1.5)	88	9.1 (2.7)	13.3 (1.6)	88	7.0 (2.2)	11.7 (1.1)	88	3.0 (2.4)	5.4 (2.9)	63	1.9 (0.7)	2.9 (1.3)	38
rA2cp248/404/1030ΔSH	4.3	13[d]	33	3.4 (0.3)	5.2 (2.6)	33	7.7 (3.0)	10.9 (4.2)	36	6.6 (2.0)	8.9 (3.2)	36	2.8 (1.0)	5.2 (3.8)	36	2.3 (0.4)	3.7 (3.0)	18
rA2cp248/404/1030ΔSH	5.3	8	100	3.3 (0.0)	7.2 (1.6)	88	6.9 (1.7)	14.2 (1.4)	100	5.0 (0.8)	10.9 (2.5)	88	3.3 (1.9)	6.7 (4.2)	63	2.2 (0.4)	4.2 (2.4)	55
Placebo[b]	...	3	33[e]	5.1 (1.6)	3.9 (1.0)	0	9.1 (1.8)	10.5 (4.1)	33	7.1 (3.6)	8.4 (3.9)	33	1.3 (0.0)	3.5 (3.0)	50	1.8 (0.0)	1.8 (0.0)	0
Placebo[c]	...	13	0	3.4 (0.3)	3.3 (0.0)	0	7.5 (2.6)	7.1 (2.2)	0	5.9 (1.5)	5.3 (1.5)	0	3.2 (1.5)	3.0 (1.2)	0	2.2 (0.8)	2.1 (0.5)	0
Infants																		
rA2cp248/404/1030ΔSH																		
First dose	4.3	16	6	7.7 (1.0)	7.0 (1.1)	0	13.6 (1.2)	12.7 (1.4)	0	13.3 (1.6)	12.4 (1.5)	0	3.0 (1.5)	3.0 (1.4)	0	2.5 (1.3)	2.6 (1.0)	6
Second dose	4.3	14	6	7.0 (1.1)	6.6 (1.2)	0	12.7 (1.4)	11.6 (0.9)	0	12.4 (1.5)	10.7 (1.3)	0	3.0 (1.4)	3.0 (1.0)	6	2.6 (1.0)	2.3 (0.8)	0
rA2cp248/404/1030ΔSH																		
First dose	5.3	16	31	7.4 (1.7)	6.8 (1.6)	0	13.4 (1.3)	12.5 (1.4)	0	12.7 (1.1)	12.1 (1.2)	0	2.1 (0.5)	3.3 (2.4)	25	2.9 (0.5)	4.4 (2.6)	31
Second dose	5.3	16	44	6.8 (1.6)	7.1 (1.5)	19	12.5 (1.4)	12.6 (1.8)	13	12.1 (1.2)	11.7 (1.4)	6	3.3 (2.4)	4.5 (3.5)	31	4.4 (2.6)	4.9 (2.7)	13
Placebo																		
First dose	...	12	0	7.7 (1.1)	7.0 (1.6)	0	13.3 (1.4)	12.7 (1.7)	0	12.4 (1.6)	11.4 (1.7)	0	2.4 (0.8)	2.4 (0.8)	0	2.6 (0.7)	2.5 (0.6)	0
Second dose	...	9	0	6.8 (1.6)	5.5 (1.8)	0	12.4 (1.5)	11.8 (1.4)	0	11.1 (1.7)	10.5 (1.7)	0	2.4 (0.8)	2.5 (0.8)	0	2.5 (0.6)	2.5 (0.6)	0

NOTE. All antibody titers are expressed as mean reciprocal log_2 values. Serum specimens were obtained before immunization and 4 weeks (for adults and respiratory syncytial virus [RSV]–seropositive children) or 8 weeks (for RSV-seronegative children) after immunization. Serum specimens were obtained from 1–2-month-old infants before the first dose was administered, before the second dose was administered, and 4 weeks after the second dose was administered.

[a] Percentage of participants who experienced a ≥4-fold increase in the indicated antibody titer.

[b] Placebo recipients in studies of rA2cp248/404ΔSH.

[c] Placebo recipients in studies of rA2cp248/404/1030ΔSH.

[d] Serum specimens were available for measurement of neutralizing antibody titer from 12 participants and for measurement of RSV F and G glycoprotein antibody titers from 11 participants; this is reflected in the given percentages of participants who experienced antibody responses.

[e] A single placebo recipient experienced increases in IgG and IgA antibody to RSV F and G glycoprotein, as measured by ELISA. This participant was inoculated in October and may have been infected with wild-type RSV before collection of the postinoculation serum specimen in December.

Table 3. Phenotype of respiratory syncytial virus (RSV) shed by recipients of rA2cp248/404/1030ΔSH and by those naturally infected with wild-type (wt) RSV.

Virus recovered from vaccine recipients	No. of participants who shed virus	No. of nasal wash specimens with virus that formed plaques at 32°C	No. of nasal wash specimens with virus that formed plaques at indicated temperature			
			35°C	36°C	37°C	38°C
Vaccine virus	45	141	48	27	1	0
wt RSV	3	9	9	9	9	9

NOTE. Original nasal wash specimens were used to perform phenotype characterization. Naturally acquired wt RSV was recovered from 2 vaccine recipients and 1 placebo recipient, as described in Results, and was included as a control. The parent virus, rA2cp248/404/1030ΔSH, can form plaques at 36°C and 37°C.

(table 1) [2, 9]. Fever, respiratory illnesses, and otitis media (OM) were defined as described elsewhere [9]. We also monitored nasal congestion in infants <6 months old [2]. In our analysis, we included nasal congestion that interfered with eating or sleeping or that resulted in obligatory mouth breathing.

Surveillance. To determine whether immunization with a live attenuated RSV vaccine was associated with enhanced disease, RSV-seronegative children and infants who received either vaccine or placebo were monitored for wt RSV infection during the subsequent RSV season [2, 9].

Virus isolation, quantitation, and phenotypic characterization. Virus was isolated from snap-frozen nasal wash specimens and identified as RSV, as described elsewhere [9]. Infectivity was quantitated by plaque assay [9]. Specimens that were negative by culture were assigned a titer of $10^{0.3}$ pfu/mL.

To determine the level of temperature sensitivity of virus present in nasal wash, specimens from recipients of rA2cp248/404ΔSH were tested for efficiency of plaque formation (EOP) at 32°C, 36°C, 37°C, 38°C, and 39°C, and specimens from recipients of rA2cp248/404/1030ΔSH were tested for EOP at 32°C, 35°C, 36°C, 37°C, and 38°C. On the basis of comparison with titers observed at 32°C, the EOP of rA2cp248/404ΔSH was ≥100-fold reduced at 38°C, and the EOP of rA2cp248/404/1030/ΔSH was ≥100-fold reduced at 35°C.

Genetic characterization. Sequence analysis was performed on 9 viruses recovered from 5 recipients of rA2cp248/404/1030/ΔSH. These viruses plaqued efficiently at 36°C and, in one case, at 37°C. Sequencing was performed directly on 1 specimen and otherwise on uncloned, passaged preparations, as described elsewhere (table 4).

Immunologic assays. Serum specimens were tested for titers of antibodies to RSV by 60% plaque reduction neutralization assay [26] and for IgG and IgA antibodies to RSV F and G glycoproteins by ELISA [9]. All titers are expressed as mean reciprocal $\log_2$ values.

Data analysis. Participants were considered to be infected if at least 1 of the following criteria was met: vaccine virus was isolated, a ≥4-fold increase in neutralizing antibody titer occurred, or ≥4-fold increases in ≥2 of the ELISA titers (serum IgG to RSV F [fusion] glycoprotein, serum IgG to RSV G [attachment] glycoprotein, serum IgA to RSV F glycoprotein, and serum IgA to RSV G glycoprotein) occurred (table 1). Mean peak titers of vaccine virus shed ($\log_{10}$ pfu/mL) were calculated for infected vaccine recipients. To calculate means, neutralizing antibody and ELISA reciprocal titers were $\log_2$ transformed. The Mann-Whitney *U* test was used to compare titers. Rates of illness were compared by Fisher's exact test (2-tailed). $P < .05$ was considered to be statistically significant.

RESULTS

Response of adults and RSV-seropositive children. In adults and RSV-seropositive children, rA2cp248/404/ΔSH and rA2cp248/404/1030ΔSH were well tolerated and highly restricted in replication (table 1). One seropositive recipient of rA2cp248/404/1030ΔSH experienced pneumonia on study day 5; this child did not have evidence of infection with vaccine virus, but enterovirus was detected on study days 4 and 5 (table 1). After vaccination, ≥4-fold increases in antibody titers rarely occurred (table 2), suggesting that these vaccines are minimally infectious or immunogenic in non–RSV-naive populations.

Response of RSV-seronegative children. In RSV-seronegative children, rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH were infectious and immunogenic. The frequency of illnesses was similar in vaccine recipients and placebo recipients (table 1). OM was observed slightly more often in RSV-seronegative vaccine recipients than placebo recipients, but it occurred sporadically throughout the study period and was not consistently associated with vaccine virus shedding. LRIs were not observed in vaccine recipients.

Although both rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH readily infected RSV-seronegative children, the level of viral replication differed significantly. The mean peak titer shed by recipients of the $10^{5.0}$-pfu dose of rA2cp248/404ΔSH was ~50-fold greater than that shed by recipients of the $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH ($10^{4.3}$ vs. $10^{2.5}$ pfu/mL, respectively; $P = .009$) (table 1), and this difference was observed throughout the study period (figure 1). The dose of rA2cp248/404/

Table 4. Phenotypic and genetic characterization of selected viruses recovered from nasal wash specimens from recipients of rA2cp248/404/1030ΔSH.

		Original nasal wash isolate						Amplified nasal wash isolate						Sequence of amplified nasal wash isolate at the indicated attenuating mutation site (gene)				
		Virus titer at indicated temperature, log_{10} pfu/mL						Virus titer at indicated temperature, log_{10} pfu/mL										
Participant or virus	Study day	32°C	35°C	36°C	37°C	38°C	Fold reduction, 36°C vs. 32°C	32°C	35°C	36°C	37°C	38°C	Fold reduction, 36°C vs. 32°C	*cp*	404 (M2)	248 (L)	1030 (L)	SH
A	10	3.2	2.5	2.3	<1.0	<1.0	7.9	6.0	NT	5.6	<1.0	<1.0	2.5	*cp*	C	**CTG/CAG Leu/Gln**	AAT Asn	−
B	5	2.3	1.3	1.5	<1.0	<1.0	6.3	6.3	5.8	5.7	<1.7	<1.7	4.0	*cp*	C	CTG Leu	AAT Asn	−
	7	3.3	2.9	2.6	<1.0	<1.0	5.0	5.7	5.6	5.2	<1.7	<1.7	3.2	*cp*	C	CTG Leu	**TAT Tyr**	−
	9	2.7	2.3	1.9	<1.0	<1.0	6.3	5.5	5.2	5.0	<1.7	<1.7	3.2	*cp*	C	CTG Leu	**TAT Tyr**	−
	10	1.3	1.0	1.5	<1.0	<1.0	<1.0	6.3	6.2	5.9	<1.7	<1.7	2.5	*cp*	C	CTG Leu	**TAT Tyr**	−
C	7	3.6	2.5	2.3	<1.0	<1.0	20.0	5.5	5.2	4.0	<1.7	<1.7	31.6	*cp*	C	CTG Leu	AAT Asn	−
D	10	3.1	2.5	2.3	1.3	<1.0	6.3	NT	NT	NT	NT	NT	NT	*cp*	C	CTG Leu	AAT Asn	−
E	12	3.1	2.9	2.8	<1.0	<1.0	2.0	6.1	NT	6.0	5.7	<1.0	1.3	*cp*	C	CTG Leu	**TAT Tyr**	−
F	11	3.5	2.5	1.7	<0.7	<0.7	63.0	6.5	5.1	4.9	3.5	<1.7	39.8	*cp*	C	CTG Leu	**AAT/TAT/CAT Asn/Tyr/His**	−
rA2cp248/404/1030/ΔSH		5.7	<1.7	<1.7	<1.7	<1.7	>10,000.0	...	...	...	...	...	...	*cp*	C	CTG Leu	AAT Asn	−
RSV A2 wt		6.6	6.6	6.6	6.5	6.6	0	...	...	...	...	...	...	wt	T	CAA Gln	TAT Tyr	+

NOTE. Participants A–E were respiratory syncytial virus (RSV)–seronegative children, and participant F was an infant. Specimens were selected for sequence analysis as described in Participants, Materials, and Methods. For participant D, virus was sequenced directly from nasal wash. All other consensus sequences were determined after 1–2 passages in Vero cells at 32°C without biological cloning (Y. Lin, A. Deatly, and W. Chen, unpublished data) [27]. After passage, the viral isolate from participant E was less temperature sensitive than the original nasal wash virus, raising the possibility that additional changes occurred during the postisolation passages. The number designations for the RSV mutants and mutations refer to the plaque numbers of the original biologically derived mutants and do not indicate amino acid or nucleotide sequence positions. Assignments that reverted to that of wild-type (wt) RSV are boldfaced. In some cases, a particular position appeared to contain a mixture of 2–3 assignments; the various assignments of these mixtures are boldfaced. +, positive; −, negative; Asn, asparagine; *cp*, cold passaged; Gln, glycine; His, histidine; Leu, leucine; NT, not tested; Tyr, tyrosine.

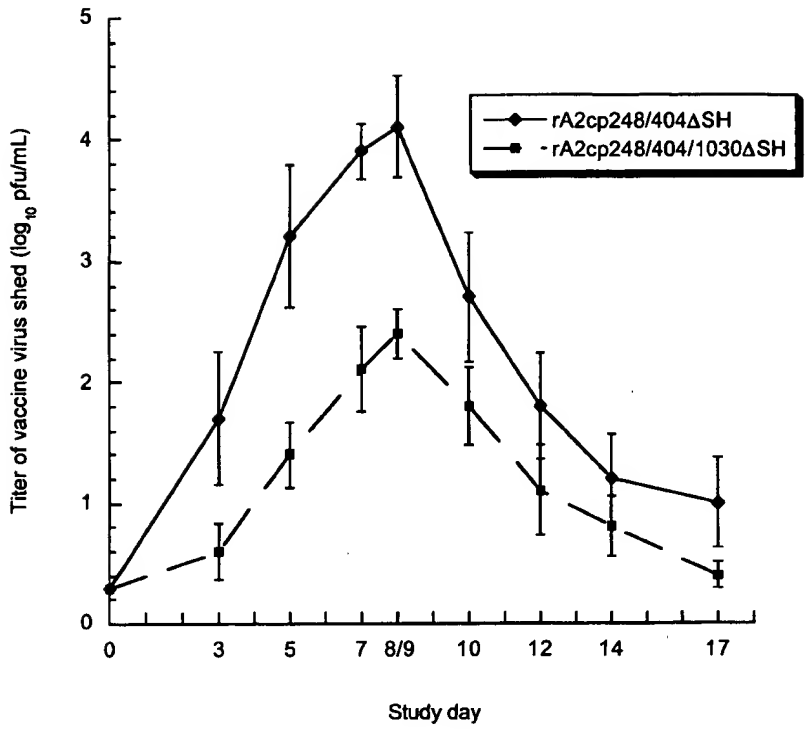

Figure 1. Replication of 10^5 pfu of rA2cp248/404ΔSH and $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH in respiratory syncytial virus–seronegative children (for each vaccine, $n = 8$). Log_{10} mean titer of vaccine virus detected in nasal wash specimens is shown for each study day; bars indicate SE.

1030ΔSH influenced the frequency of infection (69% for the $10^{4.3}$-pfu dose vs. 100% for the $10^{5.3}$-pfu dose) but not the mean peak titer shed (table 1).

Despite differences in replication, both vaccines were immunogenic in RSV-seronegative children. Eighty-eight percent of recipients of the $10^{5.0}$-pfu dose of rA2cp248/404ΔSH had ≥4-fold increases in neutralizing antibody and RSV F and G antibody titers (table 2); similarly, 100% of recipients of the $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH developed antibody responses, with mean postvaccination titers similar to those achieved with rA2cp248/404ΔSH (table 2). The dose of rA2cp248/404/1030ΔSH also influenced the frequency and magnitude of the antibody response: only 4 of 12 RSV-seronegative children who received the $10^{4.3}$-pfu dose developed a neutralizing antibody response, compared with 7 of 8 who received the $10^{5.3}$-pfu dose ($P = .03$), and the postvaccination mean reciprocal log_2 titers were 5.2 and 7.2, respectively. This difference can be explained by the increased rate of infection with vaccine virus among the recipients of the $10^{5.3}$-pfu dose and suggests that increases in dose may be one strategy for enhancement of the immune response to highly attenuated RSV vaccines.

Response of infants. rA2cp248/404ΔSH was not evaluated further because its level of replication in RSV-seronegative children was similar to that of *cpts*248/404, which caused congestion in infants [2]. In contrast, replication of rA2cp248/404/1030ΔSH was highly restricted in RSV-seronegative children, making it suitable for evaluation in infants. Two doses of either $10^{4.3}$ or $10^{5.3}$ pfu of rA2cp248/404/1030ΔSH or of placebo were administered to infants. Of the 44 infants enrolled, 2 were withdrawn before the second $10^{4.3}$-pfu dose of vaccine was administered, and 3 were withdrawn before the second dose of placebo was administered.

Mild illnesses occurred frequently and at similar rates in infants who received vaccine or placebo (table 1). Rates of illness were highest in recipients of the first $10^{4.3}$-pfu dose (69%) (table 1), but illnesses occurred at similar rates in infected and uninfected vaccine recipients (70% vs. 67%, data not shown) and were not temporally associated with viral replication. LRI was observed in 2 infants who received vaccine: rhinovirus was recovered from 1 infant with bronchiolitis (study days 4–14), and parainfluenza virus type 3 was recovered from 1 infant with pneumonia (study days 5–12). Neither infant shed vaccine virus.

As was observed for the RSV-seronegative children, replication of rA2cp248/404/1030ΔSH was highly restricted in infants—mean peak titers after the first dose of vaccine was administered were $10^{2.4}$ pfu/mL for recipients of the $10^{4.3}$-pfu dose and $10^{3.5}$ pfu/mL for recipients of the $10^{5.3}$-pfu dose (table 1). Increasing the dose increased the percentage of infected infants, from 63% to 94%; similar increases were observed for the RSV-seronegative children (table 1).

Despite the infectivity of the higher dose of rA2cp248/404/1030ΔSH in infants, the antibody responses observed were not consistent: 44% of infants developed IgA antibody responses after two $10^{5.3}$-pfu doses of vaccine, whereas 100% of RSV-seronegative children developed IgA or IgG antibody responses after a single $10^{5.3}$-pfu dose ($P = .009$). However, the antibody response in infants did not predict resistance to infection with the second dose, as the rate of infection and the magnitude of vaccine virus shed were lower after the second dose than after the first dose. These differences were significant in infants administered the $10^{5.3}$-pfu dose (rate of infection, 94% vs. 44% with respect to the first and second doses [$P = .006$]; mean peak titer, $10^{3.5}$ vs. $10^{1.3}$ pfu/mL with respect to the first and second doses [$P < .001$]) (table 1 and figure 2). These data indicate that rA2cp248/404/1030ΔSH induced immune responses in these young infants but that the mediators of this immunity remain to be identified.

Surveillance. RSV-seronegative children and infants enrolled in the present vaccine studies also participated in surveillance for RSV disease during the winters after vaccination. Enhanced disease was not observed when children and infants initially infected with vaccine virus were naturally infected with wt RSV.

Phenotypic and genetic analysis. rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH are *ts* viruses, with ≥100-fold reductions in EOP at 38°C and 35°C, respectively. In 42 nasal wash specimens from 8 recipients of rA2cp248/404/ΔSH, plaques were not detected at 39°C (data not shown), indicating stability of the *ts* phenotype. One hundred forty-one nasal wash specimens from 45 recipients of rA2cp248/404/1030ΔSH contained virus that formed plaques at 32°C (table 3). Of these specimens, 48 contained virus that formed plaques at 35°C, 27 contained virus that formed plaques at 36°C, and 1 contained virus that formed plaques at 37°C (table 3). In contrast, 9 specimens from 3 children naturally infected with wt RSV contained virus that formed plaques at 35°C, 36°C, 37°C, and 38°C (table 3).

The kinetics of the appearance of vaccine-derived viruses that formed plaques at 35°C, 36°C, and 37°C are shown in figure 3. These viruses were not detected before study day 5 (figure 3*A–E*), suggesting that they were unlikely to be present in the vaccine administered to study participants. Shedding of these viruses occurred during peak viral replication, and they sometimes predominated (figure 3*B–D*). However, shedding of these viruses diminished in parallel with the shedding of *ts* virus. Viruses that formed plaques at 36°C were present at $\leq 10^{3.0}$ pfu/mL, and virus that formed plaques at 37°C was present in a single child on a single day, at a titer of $10^{1.3}$ pfu/mL (figure 3*D*). In contrast, virus from a child naturally infected with wt RSV grew at titers of $10^{5.0}$–$10^{6.8}$ pfu/mL at all temperatures and on all study days tested (figure 3*F*).

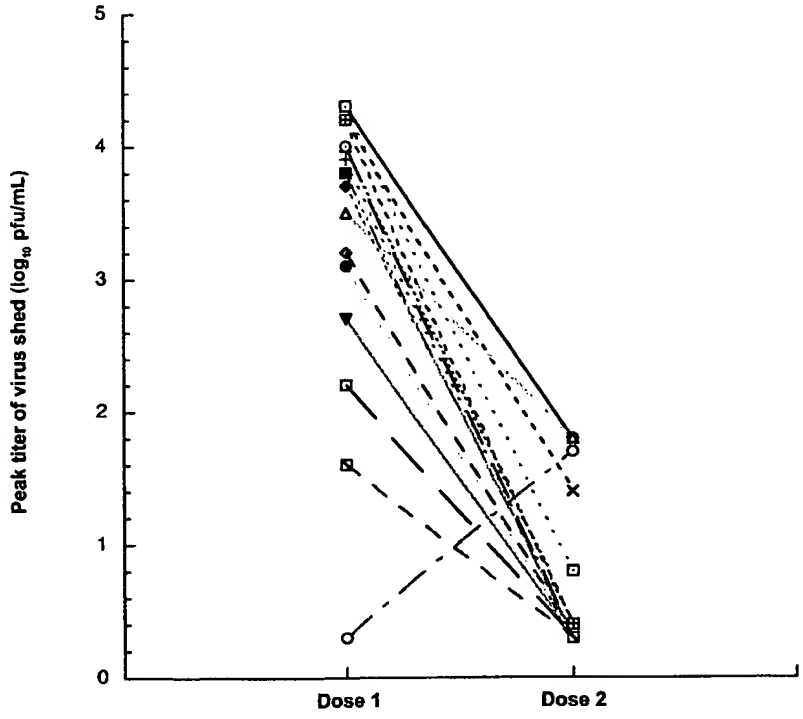

Figure 2. Relationship, in young infants, between peak virus shedding after administration of the first $10^{5.3}$-pfu dose of rA2cp248/404/1030ΔSH and peak virus shedding after administration of the second dose. The 2 doses of vaccine were administered 4–8 weeks apart, beginning at 4–12 weeks old.

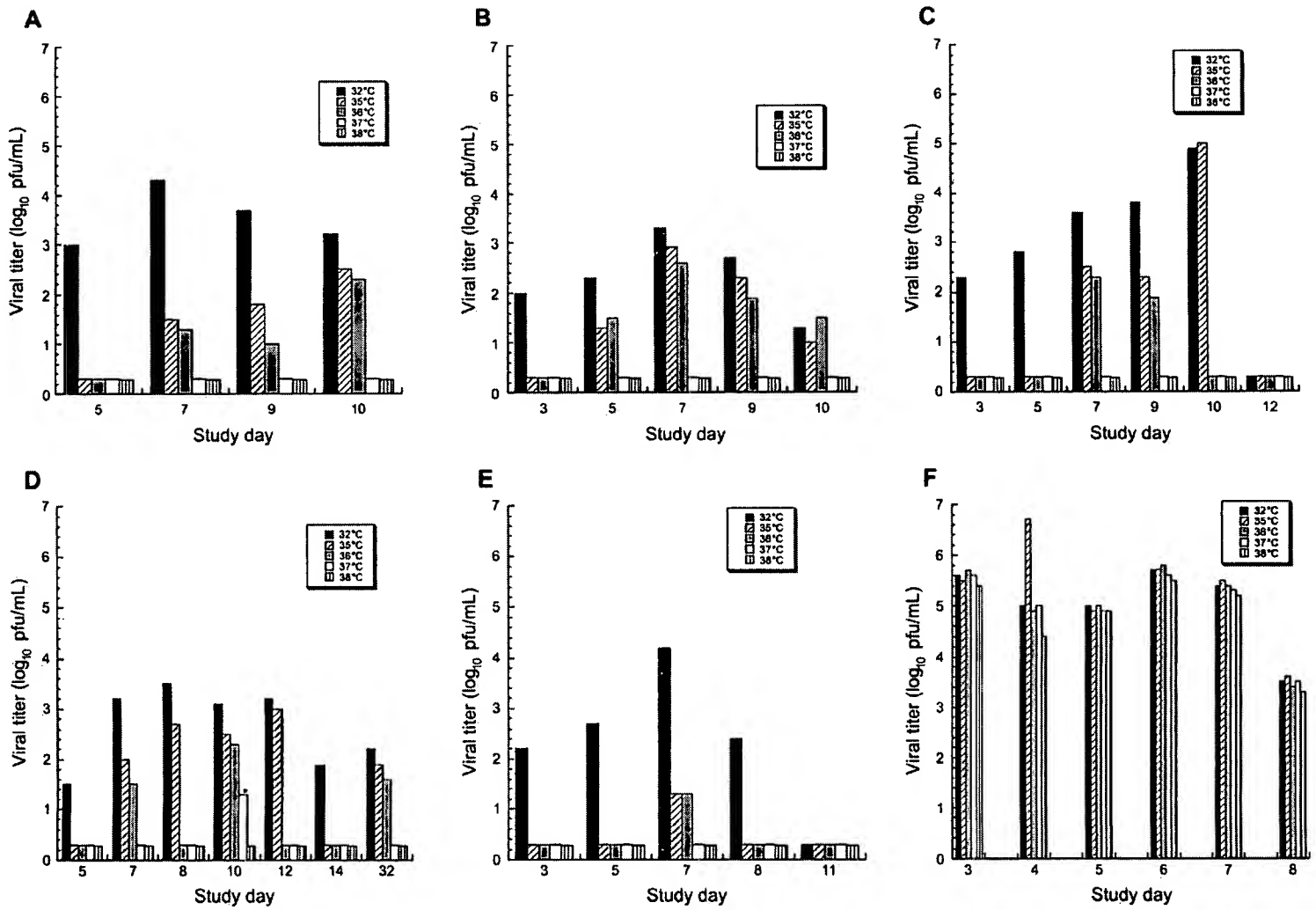

Figure 3. Phenotypic characterization of rA2cp248/404/1030ΔSH shed by 5 respiratory syncytial virus (RSV)–seronegative children *(A–E)* and of wild-type (wt) RSV shed by a naturally infected seronegative child (F) on the indicated study day. For each virus present in nasal wash specimens, efficiency of plaque formation at 32°C, 35°C, 36°C, 37°C, and 38°C is shown. Panel E depicts a representative pattern of plaque formation by virus that maintained the temperature-sensitive phenotype of the input vaccine; this was the predominant pattern observed. Panels A–D depict the pattern of shedding in which a large proportion of virus formed plaques at 35°C or 36°C (for 2 children, data not shown). A single nasal wash specimen (*), shown in panel D, contained vaccine virus that formed plaques at 37°C. Panel F depicts the pattern of plaque formation of wt RSV, for which efficient replication was observed at all temperatures, including 37°C and 38°C. Panels A–D correspond with participants A–D in table 4.

The genetic characterization of selected viruses is shown in table 4. The 5 *cp* mutations, the 404 mutation, and the ΔSH mutation were present in all isolates tested, demonstrating the stability of these mutations after replication in RSV-naive children. In 5 instances, single nucleotide substitutions were observed at either the 248 or 1030 codons, with reversion to the wt coding assignment. A sixth isolate had a mixed population of nucleotides at the 1030 codon (table 4). Each isolate had nucleotide substitutions at no more than 1 codon.

In some isolates, genetic changes were not detected, despite alteration in *ts* phenotype (table 4). In these isolates, it is likely either that mutations occurred at the known attenuating sites, but in a proportion of the uncloned virus population that was insufficient to be detected by sequence analysis, or that suppressor mutations occurred elsewhere in the genome.

DISCUSSION

Recovery of infectious virus from cDNA clones of RSV [17] has profoundly influenced the development of live attenuated RSV vaccines, because it is now possible to develop new vaccine candidates by introducing combinations of attenuating mutations into rRSV by direct manipulation of the DNA intermediate. The effect of each mutation cannot be predicted precisely, because the phenotype associated with an individual mutation is not always additive in the context of other mutations. This was observed in the present study, in which deletion of the SH gene did not further attenuate *cpts*248/404. However, incremental increases in attenuation can be achieved by including additional mutations, as was observed with the addition of the 1030 mutation to rA2cp248/404ΔSH and with other vaccine candidates under clinical development [28]. The flexibility of this technology suggests that future live RSV vaccine candidates will be developed by means of recombination techniques rather than the classical methods of serial cold passage and chemical mutagenesis [28].

Here, rA2cp248/404ΔSH was evaluated in adults and in RSV-seropositive and -seronegative children. It was minimally infectious in adults and seropositive children, which was expected on the basis of our experience with *cpts*248/404 [2, 29]. Indeed, previous studies have suggested that minimal infectivity in these individuals is a necessary prerequisite for evaluation in RSV-naive infants and children [9]. However, the mean peak titer of rA2cp248/404ΔSH shed by RSV-seronegative children was $10^{4.3}$ pfu/mL, which is comparable to that observed in seronegative recipients of *cpts*248/404 [2]. Thus, in the context of the *cpts*248/404 mutations, deletion of the SH gene does not further attenuate RSV. Because the level of replication is a useful predictor of attenuation for live respiratory viral vaccines [28], it appeared unlikely that rA2cp248/404ΔSH would be more attenuated in young infants than *cpts*248/404 [2]. For this reason, rA2cp248/404ΔSH was not evaluated in young infants.

In contrast, replication of rA2cp248/404/1030ΔSH was significantly restricted in RSV-seronegative children, with a mean peak titer of $10^{2.4}$–$10^{3.5}$-pfu/mL in nasal wash specimens. These data indicated that the 1030 mutation conferred substantial attenuation in the context of the *cp*, 248, 404, and ΔSH mutations and that rA2cp248/404/1030ΔSH was sufficiently attenuated to merit evaluation in young infants. Excess respiratory and febrile illnesses were not observed in infants infected with vaccine virus. Specifically, the clinically significant nasal congestion observed after vaccination with *cpts*248/404 was not observed in infants who received rA2cp248/404/1030ΔSH. Also, the level of replication of rA2cp248/404/1030ΔSH was comparable in infants and RSV-seronegative children, indicating that replication can occur in the upper respiratory tract even in the presence of maternally derived antibody. This finding is consistent with those of previous studies of live attenuated influenza, parainfluenza, and RSV vaccines [2, 30–33] and suggests that this outcome should be expected when these vaccines are evaluated in infants.

rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH induced high titers of RSV antibodies in seronegative children. In contrast, only a minority of infants developed antibody responses to rA2cp248/404/1030ΔSH. We previously showed that a majority of infants developed IgA antibody responses after vaccination with *cpts*248/404 [2], suggesting that the decreased antigenic load associated with the highly restricted replication of rA2cp248/404/1030ΔSH may have diminished the response in this age group. Of note, IgA antibody responses in infants were directed toward RSV F and G glycoproteins with equal frequency, whereas studies in infants with *cpts*248/404 showed responses primarily directed toward RSV G glycoprotein [2]. Further studies will be needed to address this inconsistency.

Despite the modest antibody responses, young infants, after receipt of the first dose of rA2cp248/404/1030ΔSH, were protected against challenge with the second dose; significant reductions in the proportion of infants shedding virus and the amount of virus shed were observed after the second dose. This protection was likely mediated by RSV-specific immune responses that we were unable to measure but that were effective in preventing infection and in clearing virus from the respiratory tract. Future efforts should be directed toward determination of the correlates of protection in young infants.

Although both rA2cp248/404ΔSH and rA2cp248/404/1030ΔSH are *ts* viruses, rA2cp248/404/1030ΔSH is more *ts* than is rA2cp248/404ΔSH. When the *ts* phenotype of viruses recovered from nasal wash specimens of children who received these vaccines was assessed, there was no evidence of reversion to wt virus. Virus present in specimens from children who received rA2cp248/404ΔSH maintained the *ts* phenotype of the parent virus (no plaques were detected at 39°C). Some change in the *ts* phenotype occurred in a fraction of the specimens obtained

from recipients of rA2cp248/404/1030ΔSH: 27 of 141 specimens contained virus that formed plaques at 36°C, and 1 specimen contained virus that formed plaques at 37°C. Genetic characterization revealed reversion of either the 248 or the 1030 mutation in several isolates, with preservation of the *cp*, 404, and ΔSH mutation in all recovered viruses.

It is perhaps not surprising that a subpopulation of viruses that could replicate at 36°C–37°C was generated after infection with rA2cp248/404/1030ΔSH, because selective pressure for generation of these viruses would exist in the upper airway. Although the clinical significance of these viruses cannot be determined from the present study, they remained highly *ts* and would likely be at least as attenuated as *cpts*248/404, which was attenuated even in young infants [2]. These viruses also retained all of the non-*ts* attenuating mutations and 2 of the 3 *ts* attenuating mutations present in the vaccine. Studies of live attenuated influenza and parainfluenza vaccines have shown that *ts* and non-*ts* mutations independently contribute to the stability of the attenuation phenotype [34–36]; therefore, it is likely that the viruses shed by recipients of rA2cp248/404/1030ΔSH would remain highly attenuated. Also, because the viruses were shed at $\leq 10^3$ pfu, they would not likely be transmitted to others, but transmissibility studies are needed to address this issue. The potential transmissibility of these highly attenuated vaccine-derived viruses should be considered in the context of wt RSV, which is highly contagious and infects virtually all children by 2 years of age [37].

These rRSV vaccine candidates are the first to be evaluated in clinical trials. rA2cp248/404ΔSH is not suitable for infants, given its level of replication in RSV-seronegative children. However, rA2cp248/404/1030ΔSH is the first RSV vaccine candidate that appears to be appropriately attenuated for young infants. rA2cp248/404/1030ΔSH shows limited phenotypic instability. It remains to be seen whether attenuating *ts* point mutations can be stabilized by means of molecular techniques [38] or whether some instability must be expected in highly *ts* live respiratory virus vaccines. In light of the limited replication after the second dose, multiple doses of rA2cp248/404/1030ΔSH might protect against RSV-associated LRI, despite the limited antibody response in young infants. Field trials should be conducted to address this issue. Strategies to augment the antibody response in infants, such as enhancement of RSV F and G glycoprotein expression by gene shift to promoter proximal positions [39], should also be explored.

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Mapping of a Region of the Paramyxovirus L Protein Required for the Formation of a Stable Complex with the Viral Phosphoprotein P

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The paramyxovirus large protein (L) and phosphoprotein (P) are both required for viral RNA-dependent RNA polymerase activity. Previous biochemical experiments have shown that L and P can form a complex when expressed from cDNA plasmids in vivo. In this report, L and P proteins of the paramyxovirus simian virus 5 (SV5) were coexpressed in HeLa T4 cells from cDNA plasmids, and L-P complexes were examined. To identify regions of the SV5 L protein that are required for L-P complex formation, 16 deletion mutants were constructed by mutagenesis of an SV5 L cDNA. Following coexpression of these L mutants with cDNA-derived P and radiolabeling with ^{35}S-amino acids, cell lysates were analyzed for stable L-P complexes by a coimmunoprecipitation assay and by sedimentation on 5 to 20% glycerol gradients. Mutant forms of L containing deletions that removed as much as 1,008 residues from the C-terminal half of the full-length 2,255-residue L protein were detected in complexes with P by these two assays. In contrast, large deletions in the N-terminal half of L resulted in proteins that were defective in the formation of stable L-P complexes. Likewise, L mutants containing smaller deletions that individually removed N-terminal regions which are conserved among paramyxovirus and rhabdovirus L proteins (domain I, II, or III) were also defective in stable interactions with P. These results suggest that the N-terminal half of the L protein contains sequences important for stable L-P complex formation and that the C-terminal half of L is not directly involved in these interactions. SV5-infected HeLa T4 cells were pulse-labeled with ^{35}S-amino acids, and cell extracts were examined by gradient sedimentation. Solubilized L protein was detected as an ~8 to 10S species, while the P protein was found as both a ~4S form (~85%) and a species that cosedimented with L (~15%). These data provide the first biochemical evidence in support of a simple domain structure for an L protein of the nonsegmented negative-sense RNA viruses. The results are discussed in terms of a structural model for the L protein and the interactions of L with the second viral polymerase subunit P.

The parainfluenza virus simian virus 5 (SV5) is a prototype of the paramyxovirus family of nonsegmented negative-sense RNA viruses. Six major viral proteins are found within the paramyxovirus particle (reviewed in reference 13). Three of these proteins, the fusion (F), hemagglutinin-neuraminidase (HN), and membrane (M) proteins, are found within or closely associated with the viral lipid envelope. The nucleocapsid protein (NP) is the most abundant of the paramyxovirus structural proteins (~2,600 copies per Sendai virion [24]). NP is tightly bound to the ~15-kb viral genomic RNA to form an RNase-resistant nucleocapsid (NC) structure. Two other NC-associated polypeptides, the phosphoprotein (P) and the large protein (L), together constitute the viral RNA-dependent RNA polymerase. Both the L and P polypeptides are required for transcription and replication of the NP-encapsidated genomic RNA to produce viral mRNAs and progeny virus genomes (4, 14, 15, 26). Although the exact role of L and P in the synthesis of viral RNA is unknown, the relative abundance of the L and P polypeptides in virions (~40 and ~300 molecules per Sendai virion, respectively [24]) and their distribution on the NC template (36) are consistent with the proposal that L acts as the catalytic subunit of the viral polymerase while P functions both in conjunction with L and in a noncatalytic role.

By contrast to the understanding of the structure and characteristics of the P protein (M_r ≈ 46,000), there is less known about the structure of the paramyxovirus L polypeptide (13). The majority of this limited information concerning the paramyxovirus L is based on either comparisons of protein sequences deduced from cDNA clones (34, 39) or analogies with the L protein of vesicular stomatitis virus (VSV), a rhabdovirus which employs a similar replication strategy (reviewed in references 1 and 2). Nearly half of the paramyxovirus and rhabdovirus genomes (>6,000 bases) is devoted to encoding the L protein. The large size of the L polypeptide chain (M_r ≈ 250,000) may reflect the presence of multiple distinct catalytic domains responsible for performing the individual steps in RNA synthesis (8, 13). In support of the proposal that L is multifunctional, both genetic and in vitro reconstitution experiments using VSV proteins have implicated the L polypeptide in RNA capping (1), methylation (17), chain initiation, elongation (16, 40), termination, and polyadenylation (20). A comparison of the deduced amino acid sequences of those viral L protein genes which have been cloned has shown that the predicted N- and C-terminal regions of these L proteins are diverse, whereas the middle region contains at least six domains of high amino acid sequence identity and spacing (32, 34, 39). Several of these conserved regions contain limited homology with amino acid motifs associated with functions in other cellular and viral proteins (34, 35). Although

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the importance of two of these conserved regions in the synthesis of viral RNA has been confirmed experimentally for the VSV L protein (5, 40), no specific function has been assigned to any region of a viral L polypeptide.

In the case of the paramyxovirus Sendai virus, recent biochemical experiments have shown that cDNA-derived P and L proteins expressed in vivo can be detected as a complex by coimmunoprecipitation and velocity sedimentation assays (18). Furthermore, the formation of an L-P complex correlates with the ability of the cDNA-derived L and P proteins to synthesize viral RNA (18). Thus, the well-established observation that both L and P polypeptides are required for viral RNA synthesis (14, 15, 28) can be explained, at least in part, by a need to form an L-P complex for polymerase activity (18). As it appears that interaction with the P protein is essential for L function, locating polypeptide domains that direct these L-P interactions is critical for our understanding of both the structure-function relationships of individual regions of the multifunctional L protein as well as the steps involved in viral replication. Mapping of an L-binding domain to a region of the VSV P protein has been reported previously (9). However, the complementary regions on an L polypeptide which are involved in interactions with P have not yet been identified. The recent finding that cDNA-derived L and P can form a complex when expressed in vivo (18) provides an opportunity to map the regions of the L protein involved in L-P complex formation. On the basis of the assumption that it is likely that a polypeptide the size of L contains distinct domains which could fold independently (29), I have carried out a deletion analysis of the SV5 L protein with the primary goal of mapping a P-binding site to a region within the L polypeptide. This report presents biochemical evidence that the SV5 L and P proteins form a complex when expressed from cDNA plasmids in vivo or during a virus infection. cDNA-derived deletion mutants of L were tested for the ability to form a complex with P protein in vivo. The results of this analysis indicate that an N-terminal region of L is important for the formation of stable L-P complexes. These data provide the first experimental evidence for a simple domain structure for a viral L protein and are discussed as part of a model that will form a basis for future mutagenesis.

MATERIALS AND METHODS

Cells and viruses. Monolayer cultures of CV1 and HeLa T4 (25) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Vaccinia virus vTF7.3 (12) was grown and titers were determined in CV1 cells. Infections with the W3A strain of SV5 (6) were as described previously (30).

Plasmid construction and deletion mutagenesis. A cDNA plasmid (pGem2-P) that encodes the SV5 P protein under control of the T7 RNA polymerase promoter (33, 41) was kindly provided by Reay Paterson, Northwestern University. The cloning and sequencing of the SV5 L protein gene have been described previously (32), and this cDNA was used as starting material for the deletion mutagenesis. To facilitate expression of the L protein from this cDNA, ATG codons which were located 5′ to that initiating the L open reading frame were removed by using 5′ mRNA-sense (bases 12 to 43) and 3′ vRNA-sense (bases 705 to 734) synthetic DNA primers in a PCR with Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) as described previously (7, 32). Briefly, the 5′ portion of the L gene (L-5′, bases 7 to 474 [32]) was modified to include both a unique 5′ *Xba*I site in the noncoding region and an *Nco*I site (CCATGG) in the sequences which encode the L protein-initiating AUG. This modified DNA fragment was then linked to the remaining L protein gene sequences (bases 475 to 6822) through the *Pml*I site at base 475. The resulting complete SV5 L protein gene was inserted into the *Xba*I and *Sal*I sites of pGem3 (Promega, Madison, Wis.) to produce pGem3-L. The orientation of the L gene was such that mRNA-sense transcripts could be produced by using the T7 RNA polymerase promoter.

Plasmids encoding L protein mutants with in-frame deletions were constructed by using standard techniques. The restriction sites illustrated schematically in Fig. 1A that were used to construct the L deletion mutants and the amino acids removed from the full-length 2,255-residue protein were as follows: mutant 1 (*Nco*I to *Dra*I), residues 2 to 101; mutant 2 *Nco*I to *Msc*I), residues 2 to 419; mutant 3 (*Dra*I to *Msc*I), residues 102 to 419; mutant 4 (*Pml*I to *Bst*EII), 151 to 815; mutant 5 (*Bgl*I to *Bgl*I), 354 to 962; mutant 6 (*Xho*I to *Xho*I), residues 623 to 1235; mutant 7 (*Bsp*EI to *Bsp*EI), residues 1146 to 2153; mutant 8 (*Bsp*EI to *Sca*I), residues 1146 to 1957; mutant 9 (*Sca*I to *Eco*RV), residues 1958 to 2074; mutant 10 (*Hpa*I to *Bsp*EI), residues 879 to 2153; mutant 11 (*Bst*EII to *Eco*RV), residues 817 to 2074; mutant 12 (*Xho*I to *Bsp*EI), residues 621 to 2153; and mutant 13 (*Hinc*II to *Bsp*EI), residues 566 to 2153. In brief, mutants 3 and 5 to 7 were constructed by digestion of pGem3-L with either *Dra*I and *Msc*I together (to create mutant 3) or with *Bgl*II (mutant 5), *Xho*I (mutant 6), or *Bsp*EI (mutant to 7) only, and the plasmid DNA was subsequently ligated. Likewise, mutants 1 and 2 were constructed by digestion of pGem3-L with *Nco*I followed by treatment with the Klenow fragment of DNA polymerase I to create a blunt-end DNA segment, which was in turn ligated to a 3′ *Dra*I (mutant 1) or *Msc*I (mutant 2) site. Mutant 4 was constructed by digestion of pGem3-L with *Bst*EII, treatment with Klenow fragment, and digestion with *Pml*I before religation. Mutants 8 to 13 were constructed by ligating the appropriate fragment containing the 5′ unique *Xba*I site and a 3′ site shown in Fig. 1A to pGem3-L digested with *Xba*I and either *Sca*I (mutant 8), *Eco*RV (mutants 9 and 11), or the *Bsp*EI site at bases 6481 to 6486 that had been blunt-ended by prior treatment with Klenow DNA polymerase (mutants 10, 12, and 13).

Mutants 14 to 16 were constructed by PCR as described previously (7) to encode deletions that precisely removed conserved domain I (amino acid residues 228 to 418), domain II (residues 510 to 621), or domain III (residues 656 to 878). To construct mutant 14, a DNA fragment containing pGem3 sequences 5′ to the L gene and L gene bases 1 to 1280 was used as template in a PCR along with 5′ and 3′ synthetic primers complementary to the T7 polymerase promoter and L protein gene bases 687 to 707 (5′-GCGGTCGACAATATTCTCAGT GTTAAATAATGC-3′), respectively. The 3′ primer was designed to introduce changes which converted bases 705 to 710 of the L cDNA clone to an *Ssp*I site (underlined). After digestion with *Ssp*I and *Xba*I, the resulting 750-bp PCR product was cloned into pGem2 for sequencing. The *Pml*I-*Ssp*I fragment (bases 475 to 707) was then excised and linked by blunt-end ligation to the *Msc*I site of the wild-type L protein gene at base 1281. To construct mutant 15, a DNA fragment containing bases 487 to 2474 was used as a template in a PCR along with 5′ and 3′ synthetic primers corresponding to bases 705 to 734 (5′-AATCATACACTAACATACAT GACCTTTGAA-3′) and the complement of bases 1533 to 1552 (5′-GAGGCCTCTCGAGGATTTGGTAGGGGGACCT G-3′), respectively. The 3′ primer was used to convert bases 1552 to 1557 to an *Xho*I site (underlined). After digestion with *Xho*I and *Bgl*II, the resulting DNA fragment was ligated into a

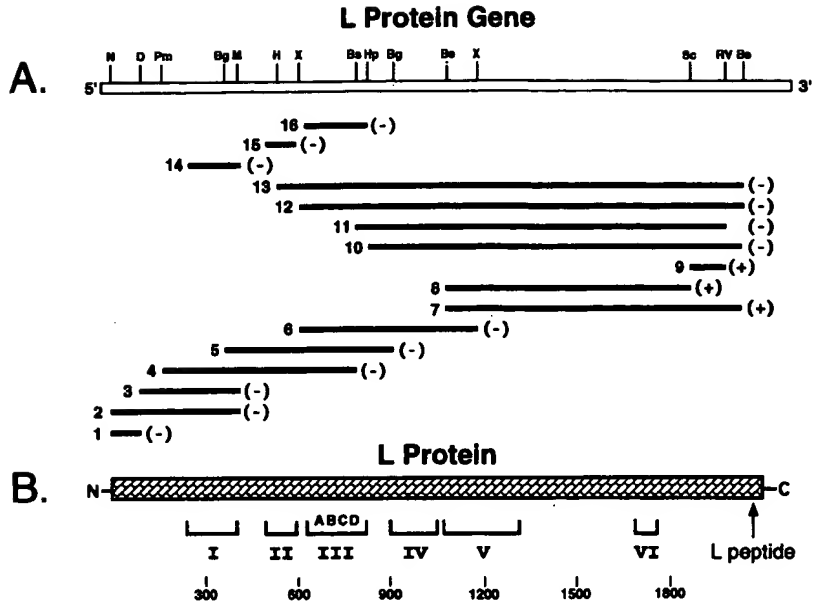

FIG. 1. Schematic diagram of the SV5 L protein gene, deletion mutants, and L protein. (A) L protein gene. The cDNA encoding the SV5 L protein is depicted as an open box, with relevant endonuclease restriction sites shown above their positions in the gene. Numbered black lines indicate portions of the L gene that were deleted to create mutants 1 to 16. (+) and (−) refer to the ability of the mutants to form a stable complex with the P protein in the two assays described in the text. Abbreviations: N, *Nco*I; D, *Dra*I; Pm, *Pml*I; Bg, *Bgl*II; M, *Msc*I; H, *Hin*cII; X, *Xho*I; Bs, *Bst*EII; Hp, *Hpa*I; Be, *Bsp*EI; Sc, *Sca*I; RV, *Eco*RV. (B) L protein. The L polypeptide is depicted as a hatched box, with brackets indicating the location of regions of high amino acid homology among L protein sequences (I to VI). ABCD denotes the location of the polymerase core domain described by Poch et al. (34, 35). The position of the synthetic peptide used to generate a antiserum to the L protein (L peptide) and amino acid numbers within the L polypeptide are indicated. N, amino terminus; C, carboxy terminus.

pGem3 vector for sequencing. A DNA fragment corresponding to bases 1153 to 1557 was excised by digestion with *Pst*I and *Xho*I and linked to the corresponding sites at bases 1153 and 1888 of the wild-type L gene. To construct mutant 16, a DNA fragment (bases 1153 to 3538) was used as a template in a PCR along with 5′ and 3′ synthetic primers corresponding to L gene bases 1303 to 1321 (5′-ATGCTTCAAAGGGTCTCAC-3′) and the complement of bases 1971 to 1991 (5′-CGCGCCC GGGAATTCAAAGTCATCACTTGG-3′), respectively. The 3′ primer was designed to introduce changes which converted bases 1986 to 1991 of the L cDNA clone to an *Eco*RI site (underlined). The resulting 680-bp DNA was digested with *Eco*RI and *Xho*I and cloned into the corresponding sites of pGem7 vector for sequencing. Plasmid DNA was digested with *Eco*RI, blunt ended with Klenow fragment, and digested with *Xho*I. The resulting 100-bp fragment was ligated into the *Xho*I and *Hpa*I sites of the wild-type L gene at bases 1888 and 2660, respectively. The nucleotide sequence of all of the above PCR-derived DNA fragments was determined by dideoxynucleotide sequencing (37).

Isotopic labeling of polypeptides, immunoprecipitation, and PAGE. Proteins were expressed in HeLa T4 cells from cDNA plasmids as described previously (31), using a modified version of the vaccinia virus/T7 RNA polymerase system of Fuerst et al. (12). Briefly, 35-mm-diameter dishes of subconfluent HeLa T4 cells were infected with vaccinia virus vTF7.3 (multiplicity of infection of ≈10) for 1 h and then transfected with pGem2-P (~2 μg) and/or pGem3-L (~2 to 6 μg) mutant plasmid, using Lipofectin reagent (12 μg in 800 μl of Opti-MEM [Gibco-BRL, Gaithersburg, Md.]) according to the manufacturer's instructions. Differing amounts of the various mutant L plasmids were used in transfections to obtain approximately the same level of L expression. Cells were radiolabeled from 11 to 12 h postinfection (hpi) with 50 to 100 μCi of Tran[^{35}S]label (ICN Radiochemicals Inc., Irvine, Calif.) per ml in Dulbecco's modified Eagle's medium lacking cysteine and methionine, washed in cold phosphate-buffered saline (PBS), and lysed in buffer containing the appropriate detergent. Proteins were radiolabeled in SV5-infected HeLa T4 cells as described previously (30). For immunoprecipitation of sodium dodecyl sulfate (SDS)-denatured proteins, radiolabeled cells were lysed in 250 μl of 1% SDS and boiled for 5 min. Cell extracts were clarified by centrifugation and diluted with 4 to 6 volumes of buffer (60 mM Tris-HCl [pH 7.4], 190 mM NaCl, 6 mM EDTA, 1.25% Triton X-100 [11]). Proteins were immunoprecipitated as described previously (23), using *Staphylococcus aureus* protein A-agarose (Gibco-BRL). The rabbit polyclonal antiserum to detergent-disrupted SV5 virus (anti-SV5 serum) has been described elsewhere (32); this serum recognizes all of the known SV5 proteins except the L polypeptide. The rabbit polyclonal L-specific (anti-L protein) serum was the kind gift of Robert Lamb, Howard Hughes Medical Institute. This serum was raised to a synthetic peptide that had been chemically linked to keyhole limpet hemocyanin. The sequence of the peptide corresponded to C-terminal amino acids 2241 to 2254 of the SV5 L protein (one-letter amino acid code, NH_2-N-I-N-E-E-I-D-R-G-I-D-G-E-E-COOH [32]). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide gels followed by fluorography (22). Autoradiograms and figure labels were electronically scanned with a Microtek Scanmaker 600ZS, using Adobe Photoshop software.

Glycerol gradient centrifugation. L-P protein complexes were analyzed by glycerol gradient centrifugation, using a

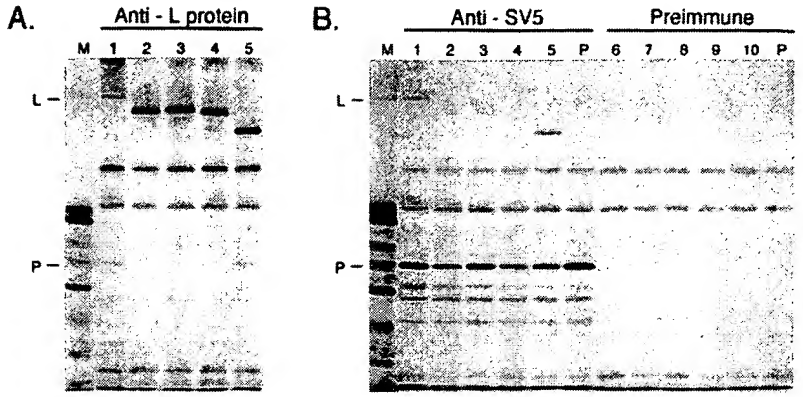

FIG. 2. Coimmunoprecipitation of cDNA-derived L and P proteins. Vaccinia virus vTF7.3-infected HeLa T4 cells were cotransfected with a plasmid encoding P protein (2 μg) along with plasmids encoding either wild-type L protein (3 μg) or L protein deletion mutants 4 to 7 (2 to 4 μg). Cells were radiolabeled from 11 to 12 hpi with Tran[^{35}S]label. Equal aliquots of detergent cell lysates were immunoprecipitated under nondenaturing conditions with either anti-L protein (A) or anti-SV5 or preimmune (B) serum before analysis by SDS-PAGE. Lanes 1 to 5 represent samples from cells cotransfected with plasmids encoding P with either wild-type L (lane 1) or L mutants 4 to 7 (lanes 2 to 5, respectively). Lanes 6 to 10 of panel B represent samples from the same lysates as lanes 1 to 5, respectively. P lanes in panel B are samples of proteins from cells transfected with P plasmid alone and immunoprecipitated with anti-SV5 or preimmune serum. M is a marker lane of SV5-infected cell polypeptides, with the positions of the wild-type L and P proteins indicated.

variation of the method described by Horikami et al. (18). Vaccinia virus vTF7.3-infected HeLa T4 cells (35-mm-diameter dishes) were transfected with plasmids encoding P and mutant L proteins as described above. Alternatively, cells were infected with SV5 (30). Cells were radiolabeled with 50 μCi of Tran[^{35}S]label per ml from 11 to 12 hpi for the vaccinia virus infection or for 30 min at 15 to 19 hpi for the SV5 infection. The cell monolayers were washed in ice-cold PBS before lysis in 0.5 ml of buffer G (25 mM Tris-HCl [pH 7.5], 0.5% Nonidet P-40 [NP-40], 250 mM NaCl, 2 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 14 mM β-mercaptoethanol). Cell lysates were clarified by centrifugation (10 min, 4°C, 160,000 × *g*, TLA100 rotor [Beckman Instruments, Palo Alto, Calif.]), and the resulting supernatant was operationally defined as the soluble fraction. An aliquot (0.4 ml) of the supernatant was layered onto a 12-ml preformed linear 5 to 20% (vol/vol) glycerol gradient in buffer G. Following centrifugation (25 h, 39,000 rpm, 5°C, SW41 rotor), 12 1-ml fractions were collected from the bottom of the tubes. Each fraction was adjusted to 0.5% SDS, boiled for 5 min, and diluted with buffer as described above. Samples were immunoprecipitated by using 8 μl (cDNA-derived proteins) or 12 μl (SV5-derived proteins) of a 1:1 mixture of anti-L protein and anti-SV5 sera before analysis of the immune complexes by SDS-PAGE. Protein standards (rabbit hemoglobin, ~4S; mouse immunoglobulin G1, 7S; potato β-amylase, 9S) were analyzed in parallel gradients, and gradient fractions were analyzed by SDS-PAGE followed by Coomassie blue staining.

Coimmunoprecipitation assays. Coimmunoprecipitation of cDNA-derived P and L proteins was carried out by a variation of the method described by Shaw et al. (38). Briefly, vaccinia virus vTF7.3-infected, cDNA-transfected cells were radiolabeled for 1 h with Tran[^{35}S]label as described above and then washed with ice-cold PBS before lysis in 1 ml of cold buffer R (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 5 mM EDTA, 0.4% deoxycholate, 1% NP-40, 0.5% bovine serum albumin, 25 mM iodoacetamide). Following centrifugation in the TLA100 rotor as described above, the supernatant was adjusted to 0.1% SDS and pretreated (1 h, 4°C) with 5 to 8 μl of preimmune serum and 50 μl of a 1:1 (vol/vol) slurry of protein A-agarose. The agarose beads were pelleted, and the resulting precleared supernatant was then incubated for 2 h at 4°C with 10 μl of anti-SV5 serum. After the addition of protein A-agarose beads, the immune complexes were washed three times at 4°C with 1 ml of ice-cold buffer R containing 0.1% SDS before analysis by SDS-PAGE. In parallel, aliquots representing one half of the cell lysate used in the coprecipitation assay were adjusted to 1% SDS, boiled, and immunoprecipitated with the anti-L protein serum.

RESULTS

Effect of L mutations on the formation of an L-P complex. It has been previously shown by using a coimmunoprecipitation assay that cDNA-derived Sendai virus L and P proteins form a complex in vivo (18). To determine if SV5 L and P proteins could also form a complex when expressed in vivo from cDNA plasmids, a similar coimmunoprecipitation assay was used as described previously (38). Monolayers of HeLa T4 cells were first infected with vTF7.3, a recombinant vaccinia virus which expresses the bacteriophage T7 RNA polymerase (12). These infected cells were then cotransfected with a cDNA plasmid encoding the SV5 L protein (pGem3-L; Fig. 1A) and a plasmid encoding the SV5 P protein (pGem2-P [41]), both of which were under control of the T7 RNA polymerase promoter. Following radiolabeling with Tran[^{35}S]label, detergent cell lysates were prepared under nondenaturing conditions described in Materials and Methods. Lysates were immunoprecipitated either with an antiserum raised to a synthetic peptide whose sequence corresponds to a C-terminal region of the SV5 L protein (anti-L protein serum; Fig. 1B) or with an antiserum raised to disrupted SV5 virus (anti-SV5 [32]). This anti-SV5 serum does not contain antibodies that recognize the L protein (data not shown).

As shown in Fig. 2, the SV5 L protein was immunoprecipitated by the anti-L protein serum from extracts of vTF7.3-infected cells that had been cotransfected with pGem3-L and pGem2-P (Fig. 2A, lane 1). Immunoprecipitation of an aliquot of the same lysate with the anti-SV5 serum showed precipitation of the P protein, along with a polypeptide that comigrated

with L (Fig. 2B, lane 1). This additional polypeptide was not found in immune complexes isolated from cells that had been transfected with the P plasmid alone (Fig. 2B, anti-SV5 panel, lane P). Neither P or L polypeptides were precipitated from these lysates with preimmune serum (preimmune lanes). For unknown reasons, the amount of P in immune complexes isolated by using the anti-L protein serum (Fig. 2A, lane 1) was always less than that of the converse precipitation using anti-SV5 serum (Fig. 2B, lane 1). As described above, the presence of L in the immune complexes isolated with the anti-SV5 serum was not due to contaminating antibodies specific for the L polypeptide. These data are consistent with coimmunoprecipitation studies reported for the Sendai virus P and L proteins (18) and indicate that SV5 cDNA-derived L and P can form a complex in the absence of other viral components.

To locate the regions of L important for interactions with the P protein, a series of L protein deletion mutants was constructed (Fig. 1). There are presently no data concerning the structure of a viral L protein from which to target these mutations. Therefore, the rationale for the location of these deletions was based on the goal of creating a panel of mutants that would span nearly the entire coding region of L. Initially, four mutant L protein genes encoding predicted internal deletions of 665, 609, 613, and 1,008 amino acids were constructed by using restriction enzyme sites within the L cDNA (Fig. 1, mutants 4 to 7). In all cases, the sequences of these altered genes retained the L protein translational reading frame, so that the mutant polypeptides could be immunoprecipitated with the anti-L protein serum. When coexpressed with P in the vaccinia virus/T7 system outlined above and immunoprecipitated from lysates with anti-L protein serum, L protein mutants 4 to 7 were detected as distinct polypeptide species (Fig. 2A, lanes 2 to 5, respectively) that migrated slightly faster than the full-length L protein (lanes 1 and M). Analysis of aliquots from these same lysates by using the anti-SV5 serum resulted in the detection of P (Fig. 2B, lanes 2 to 5), along with additional nonspecific polypeptides that were also seen when preimmune serum was used (lanes 6 to 10). Of the four altered L polypeptides tested, mutant 7 was the only L-specific polypeptide detected in immune complexes with P under these conditions at levels comparable to that obtained with the full-length L (Fig. 2B, lane 5). Immunoprecipitation of lysates from cells coexpressing L mutants 4 to 6 with P (Fig. 2B, lanes 2 to 4, respectively) resulted in a pattern similar to that found when P was expressed alone (anti-SV5 panel, lane P). Only very small amounts of mutants 4 to 6 were found in the immune complexes, and these mutants were never detected in the immune complexes at levels comparable to that found for wild-type L or mutant 7. Taken together, these data indicate that an L mutant lacking 1,008 C-terminal residues from the 2,255-residue full-length protein (mutant 7) is still capable of forming a stable L-P complex by this coimmunoprecipitation assay, but large deletions in the N-terminal half of L (mutants 4 to 6) result in proteins that are defective in these interactions.

The L and P proteins do not coimmunoprecipitate from mixed lysates of cells separately transfected with P and L plasmids. A series of mixing experiments was carried out to determine if the formation of the SV5 L-P complex described above required the coexpression of these polypeptides in the same cell. Separate dishes of vTF7.3-infected HeLa T4 cells were individually transfected with plasmid DNA encoding P, HN, L, or mutant L protein. After radiolabeling with Tran[^{35}S]label, cell lysates were prepared under nondenaturing conditions and mixed prior to immunoprecipitation with the anti-SV5 serum. As shown in Fig. 3, when lysates of cells

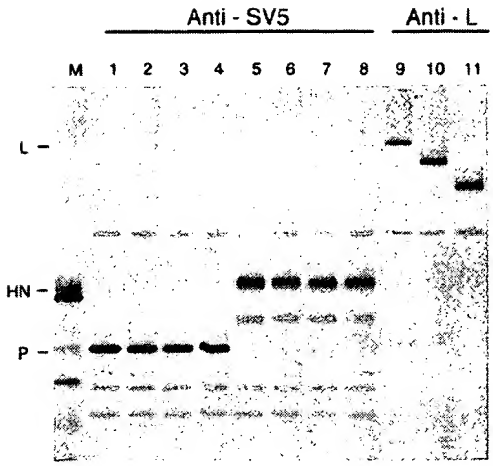

FIG. 3. The L and P proteins do not coimmunoprecipitate from mixed lysates of cells separately transfected with P and L plasmids. Separate dishes of vTF7.3-infected HeLa T4 cells were mock transfected or individually transfected with plasmids encoding either the P protein (2 μg), HN protein (5 μg), L protein (5 μg), or L protein mutant 5 or 7 (5 μg). Following a 1-h radiolabeling period with Tran[^{35}S]label, cell lysates were prepared under nondenaturing conditions described in Materials and Methods. Equal aliquots of clarified cell lysate expressing radiolabeled P protein (lanes 1 to 4) or HN protein (lanes 5 to 8) were mixed and held at 4°C for 3 h with mock-transfected cell lysates (lanes 1 and 5) or with lysate from cells expressing radiolabeled L protein (lanes 2 and 6), L protein mutant 5 (lanes 3 and 7), or L protein mutant 7 (lanes 4 and 8). After immunoprecipitated with the anti-SV5 serum, proteins were analyzed by SDS-PAGE. Lanes 9 to 11 depict proteins immunoprecipitated with the anti-L protein serum from SDS-denatured extracts of cells expressing wild-type L protein (lane 9) or L protein mutant 5 (lane 10) or 7 (lane 11). M is a marker lane of SV5-infected cell polypeptides.

that had separately expressed P protein and L protein were mixed and immunoprecipitated, the proteins isolated (lane 2) were very similar to those seen from mixed lysates of cells expressing P and mock-transfected cells (lane 1). Thus, L protein was not detected in immune complexes from these mixed lysates, even though the L protein had been synthesized at significant levels (lane 9). The L protein was not precipitated by the anti-SV5 serum when expressed alone and cell lysates were mixed with lysates from cells separately expressing cDNA-derived HN protein (compare lanes 5 and 6) or M protein (data not shown). Likewise, the proteins immunoprecipitated from mixed lysates of cells separately expressing either L mutant 5 or 7 (Fig. 3, lanes 10 and 11) with lysates from cells expressing P (lanes 3 and 4) or HN (lanes 7 and 8) were the same as that found for mock-transfected cells. Together, these data supporting the contention that the presence of L in the immune complexes described above is due to interactions with P, and this conclusion is further supported by the results of glycerol gradient sedimentation analysis described below. Furthermore, these data indicate that the formation of the L-P complex with cDNA-derived proteins appears to require or be enhanced by coexpression of the two proteins in the same cell. This is a characteristic previously described for the Sendai virus P and L proteins (18) but different from that of the VSV proteins (e.g., references 5 and 9).

Effect of N- and C-terminal L mutations on formation of L-P complexes. Five additional L protein deletion mutants were constructed to confirm the conclusions presented above and to

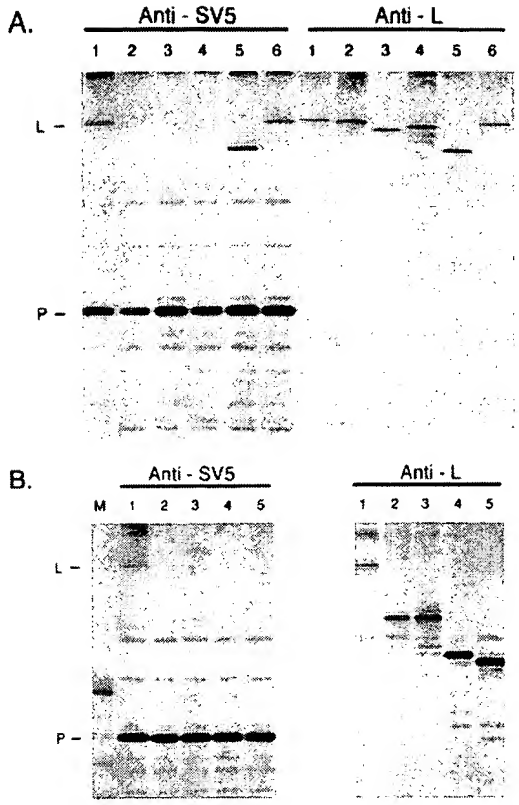

FIG. 4. Effect of L protein N- and C-terminal deletions on the coimmunoprecipitation of L with P protein. (A) Effects of N- and C-terminal deletions. HeLa T4 cells infected with vTF7.3 were cotransfected with 2 μg of a plasmid encoding P protein along with 6 μg of either wild-type L (lane 1), N-terminal L mutants 1 to 3 (lanes 2 to 4), or C-terminal L mutants 8 and 9 (lanes 5 and 6). After radiolabeling of the cells from 11 to 12 hpi with Tran[^{35}S]label, proteins were immunoprecipitated from cell lysates with the anti-SV5 serum under nondenaturing conditions (lanes 1 to 6, anti-SV5 panel) and analyzed by SDS-PAGE. Likewise, one half of the material processed for the samples depicted in the anti-SV5 panel was denatured with SDS, diluted with buffer, and immunoprecipitated with the anti-L protein serum (anti-L panel). The positions of L and P polypeptides are indicated. (B) Effect of progressive C- to N-terminal L protein deletions. vTF7.3-infected HeLa T4 cells were cotransfected with plasmids encoding P and wild-type L (lane 1) or L mutants 10 to 13 (lanes 2 to 5, respectively). Radiolabeled cell lysates were prepared and immunoprecipitated with the anti-SV5 serum (left panel) or with the anti-L protein serum (right panel) as described above. M is a marker lane of SV5-infected cell polypeptides.

further determine the effect of smaller L deletions on the formation of stable L-P complexes. N-terminal residues 2 to 101, 2 to 419, and 102 to 419 were deleted to create mutants 1 to 3, respectively (Fig. 1). Likewise, C-terminal residues 1146 to 1957 and 1958 to 2074 were deleted to create mutants 8 and 9 (total of 2,255 L residues). Plasmids encoding these altered L proteins were cotransfected into vTF7.3-infected HeLa T4 cells along with a plasmid encoding P. After radiolabeling of the cells with Tran[^{35}S]label, cell lysates were immunoprecipitated with the anti-SV5 serum under nondenaturing conditions; alternatively, an aliquot of the lysate was denatured and immunoprecipitated with the anti-L protein serum. As shown in Fig. 4A, L mutants 1, 2, 3, 8, and 9 were synthesized as major polypeptide species (anti-L panel, lanes 2 to 6, respectively) that migrated faster than wild-type L (anti-L panel, lane 1). Immunoprecipitation of aliquots of these same lysates with anti-SV5 serum under coimmunoprecipitation conditions showed wild-type L protein in immune complexes with P (anti-SV5 panel, lane 1) as described above. Likewise, C-terminal deletion mutants 8 and 9 were detected in immune complexes at levels that closely matched that found for the full length L (anti-SV5 panel; compare lane 1 with lanes 5 and 6). In contrast to these C-terminal mutants, only very small or undetectable amounts of the N-terminal deletion mutants 1 to 3 were reproducibly detected in these immune complexes with P (anti-SV5 panel, lanes 2 to 4).

To further define the C-terminal boundary of L required for stable interactions with P, the smallest L protein which bound to P in these assays (mutant 7) was used as starting material from which four additional mutants with progressive deletions in a C- to N-terminal direction were constructed (mutants 10 to 13; Fig. 1). Mutants were designed to contain deletions that would flank the C-terminal side (mutant 10 and 12) or delete into (mutant 11 and 13) two regions of L that are homologous among all viral L protein sequences (domains II and III, respectively [39]). When coexpressed with P, these mutants were readily detected in cell lysates by using the anti-L protein serum (Fig. 4B, anti-L panel, lanes 2 to 5) at levels that were consistently higher than that of wild-type L (anti-L panel, lane 1). However, the proportion of radiolabeled mutant proteins found in immune complexes isolated with the anti-SV5 serum (anti-SV5 panel, lanes 2 to 5) relative to the level of expression of the mutants (anti-L panel, lanes 2 to 5) was reproducibly much less than that found for wild-type L (compare lanes 1 of the two panels). Each of these L deletion mutants still contains 102 C-terminal residues which include the segment recognized by the antipeptide antibodies. Thus, the data from these coprecipitation assays suggest that L sequences located between C-terminal residues 1146 and 2153 (those deleted to create mutant 7) are not required for the formation of stable L-P complexes. In contrast, alterations in the N-terminal half of the SV5 L results in polypeptides which are defective in L-P interactions.

Glycerol gradient sedimentation analysis of L-P complexes. The formation of a complex between cDNA-derived P and L proteins demonstrated above by coimmunoprecipitation was confirmed by glycerol gradient sedimentation analysis. HeLa T4 cells infected with vTF7.3 were transfected with plasmids encoding L and P either alone or in combination, and cells were radiolabeled with Tran[^{35}S]label. Soluble cell lysates were sedimented on linear 5 to 20% glycerol gradients, and fractions from the gradient were denatured with SDS, diluted, and immunoprecipitated with a mixture of anti-L protein and anti-SV5 serum. When expressed in the absence of P, the cDNA-derived L protein was detected in fractions 1 to 5 near the bottom of the gradient, with a peak centered around fraction 4, and this sedimentation was found to correspond to ~8 to 10S (Fig. 5A). A second protein of unknown origin (M_r ≈ 110,000) was seen in fractions 6 and 7 which may represent a vaccinia virus-specific polypeptide. When expressed in the absence of L, the P protein sedimented as a major species centered around fraction 8 (~4S; Fig. 5B). In contrast to the results obtained when P was expressed alone, coexpression of P with L gave a unique sedimentation profile (Fig. 5C). The L protein was again detected as a ~8 to 10S species (Fig. 5C, lanes 3 and 4), but the P protein was now present as two forms: one which sedimented like that seen when P was expressed alone (~4S), and a second form which cosedimented to the same portion of the gradient as the L protein (~8 to 10S;

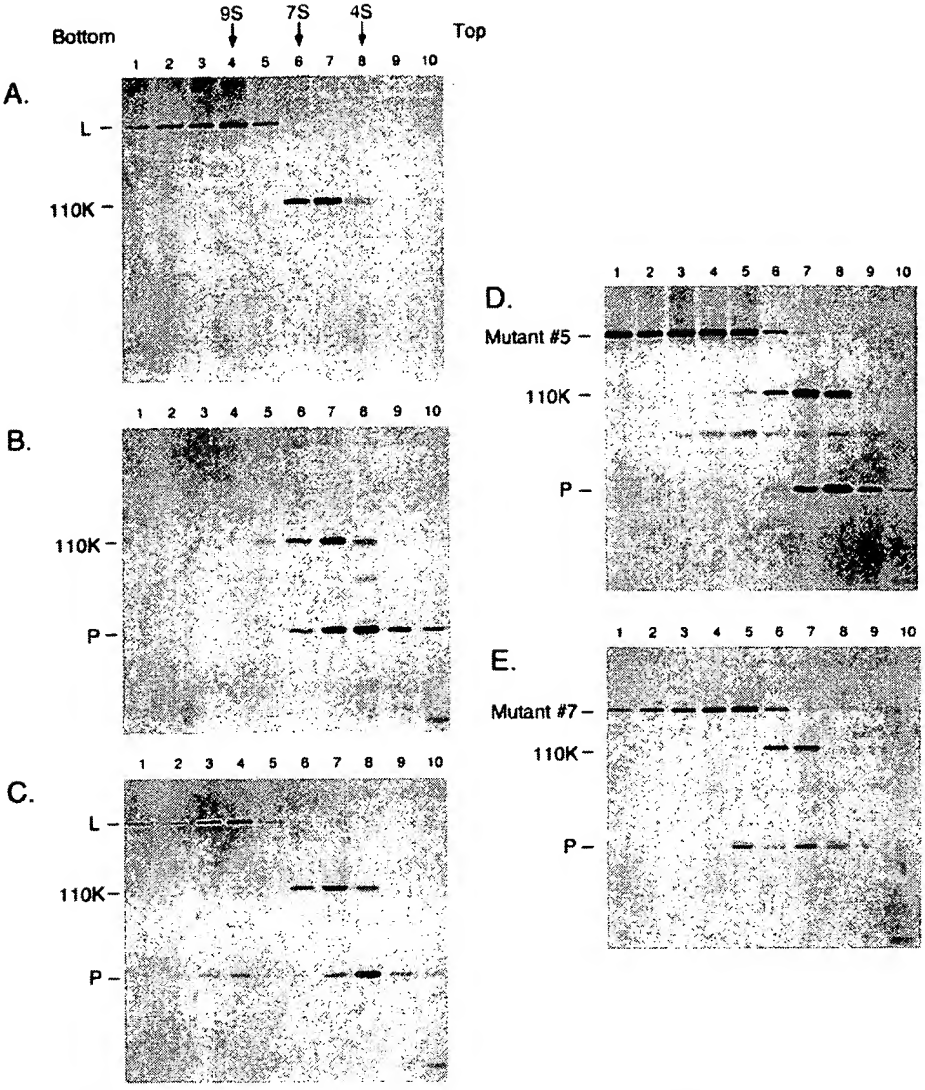

FIG. 5. Glycerol gradient sedimentation analysis of cDNA-derived L-P complexes. vTF7.3-infected HeLa T4 cells were transfected alone or in combination with plasmids encoding the P protein (4 μg), wild-type L, or L protein mutant 5 or 7 (6 μg of each). Cells were radiolabeled from 11 to 12 hpi with Tran[^{35}S]label, and soluble cell lysates were analyzed by centrifugation through linear 5 to 20% glycerol gradients as described in Materials and Methods. Fractions collected from the bottom of the gradient were denatured with SDS and immunoprecipitated with a mixture of anti-L protein and anti-SV5 sera before analysis by SDS-PAGE. Shown are gradient profiles of polypeptides from cells transfected with plasmids encoding L protein only (A), P protein only (B), L and P proteins (C), L mutant 5 and P protein (D), or L mutant 7 and P protein (E). The position of a 110-kDa protein of unknown origin is marked (110 K). Centrifugation was from right (Top) to left (Bottom), and gradient fractions are numbered. Arrows indicate the positions of 9S, 7S, and 4S sedimentation markers that were analyzed in a parallel gradient.

fractions 3 and 4). Thus, these sedimentation data demonstrate that cDNA-derived L and P proteins can form an ~8 to 10S complex in vivo in the absence of other viral components, a result which is consistent with data obtained by Horikami et al. (18) for Sendai virus L and P proteins.

As a second assay to determine if the L protein deletion mutants described above interact with P, each of the 13 L protein mutants was coexpressed in HeLa cells along with P, and cell lysates were analyzed by glycerol gradient sedimentation. Two representative examples of the results obtained from this analysis are shown in Fig. 5D and E. When coexpressed with L mutant 5, the P protein was found as a ~4S peak (Fig. 5D), with no faster-sedimenting P protein detected. This profile matched that seen when P was expressed by itself (Fig. 5B). The gel depicted in Fig. 5D was deliberately overexposed but did not reveal any P protein sedimenting in the ~8 to 10S region of the gradient. In contrast, analysis of lysates from cells coexpressing P with L deletion mutant 7 (Fig. 5E) revealed two distinct forms of P, one of which sedimented as ~4S (fractions 7 and 8), and a faster-sedimenting form was found in the same peak fraction as L mutant 7 (fraction 5). The slightly slower sedimentation of the mutant 7-P complex (Fig. 5E, lane 5) relative to that of the wild-type L–P complex (Fig. 5C, lane 4) may reflect the fact that this altered L protein is approximately half the size of full-length L. When expressed with P and analyzed by gradient centrifugation, each the L deletion mu-

tants produced a sedimentation profile that was similar to one of those shown in Fig. 5C, D, or E.

Alignments of L protein sequences have identified regions of high amino acid homology (34, 39). Three additional L protein mutants were constructed to examine the effects of small deletions in three of these conserved N-terminal domains on the formation of L-P complexes. On the basis of the locations of these conserved regions in the SV5 L protein, DNA segments encoding conserved domains I, II, and III were individually deleted from the SV5 cDNA to create mutants 14 to 16, respectively (see Materials and Methods). After coexpression of mutants 14 to 16 in HeLa T4 cells with P, lysates were assayed by gradient sedimentation for the presence of L-P complexes as described above. These three L mutants produced an L-P sedimentation profile matching that shown in Fig. 5D (data not shown) and thus were defective in the formation of stable ~8 to 10S complexes with P. Collectively, the ability of each altered L polypeptide to reproducibly interact with P in both the coimmunoprecipitation and cosedimentation assays was determined. From these data, the L mutants were scored as positive or negative for stable binding to P protein, and the summarized results are shown in Fig. 1. These results support the conclusion that regions of L important for the formation of stable SV5 L-P complexes reside in the N-terminal half of the L polypeptide.

Sedimentation analysis of L-P complexes from SV5-infected cells. Although cDNA-derived L and P can form an ~8 to 10S complex in vivo (18) (Fig. 5), it was unclear whether a similar complex is formed in SV5-infected cells. The analysis of paramyxovirus-infected cell lysates is complicated by the fact that it is likely that newly synthesized L protein rapidly associates with the viral NC structure (21, 36). Both L and P can be removed from NCs by high-salt conditions, but it is not known if the SV5 L-P complex is stable in high salt. As a first step in addressing this question, a titration experiment was carried out to determine the salt concentration needed to solubilize L from virus-infected cells. HeLa T4 cells infected with SV5 were radiolabeled for 30 min at 19 hpi with Tran[^{35}S]label, and cell lysates were prepared by using nonionic detergent and increasing salt concentrations. Samples were centrifuged at speeds sufficient to pellet viral nucleocapsids (10). Aliquots representing equal fractions of the resulting pellet and supernatant were solubilized by boiling in SDS, and samples were immunoprecipitated with the anti-L protein or anti-SV5 serum. As shown in Fig. 6A, the majority of the L protein detected with anti-L protein serum was found in the pellet fraction when cell lysates were prepared with no additional salt present (0 lanes). However, cell lysis with detergent buffer containing progressively higher concentrations of salt resulted in a larger fraction of L recovered in the supernatant. Surprisingly, detergent buffer containing as little as 125 mM NaCl resulted in the solubilizing of >50% of the pulse-radiolabeled L protein from the cell lysates (Fig. 6A; compare lanes P and S, 125 mM NaCl). Nearly all of the pulse-radiolabeled L could be recovered in the supernatant fraction by using buffer with 500 mM NaCl, but a significant amount of this protein was detected as an aggregate near the top of the gel (500 mM, S lane). The salt-dependent fractionation profile of the pulse-radiolabeled P protein in these same lysates was similar to that of L (Fig. 6B) except that 250 mM NaCl was required to solubilize >50% of the newly synthesized P protein.

To determine if the solubilized P and L proteins could be found together in a distinct complex, radiolabeled SV5-infected cell lysates prepared by using 250 mM NaCl were analyzed by sedimentation through 5 to 20% glycerol gradients. Aliquots from fractions of the gradient were immunoprecipitated with a mixture of anti-SV5 and anti-L protein sera before analysis by SDS-PAGE. The majority of the radiolabeled viral polypeptides, including the NP, M, and P proteins, sedimented slightly slower than the ~4S marker (Fig. 7, lanes 8 to 10). In contrast, the L protein was detected in the bottom one-third of the gradient as a major protein species that sedimented as an ~8 to 10S particle (lanes 4 and 5). Only trace amounts of L were recovered in the pellet at the bottom of the centrifuge tube (data not shown), suggesting that under these conditions the L protein had not formed insoluble aggregates. While the majority of P was found in the ~4S portion of the gradient with the NP protein (lanes 8 to 10), a smaller amount was detected in the same fractions as the L protein (lanes 4 and 5). Quantitation of the distribution of P in the gel depicted in Fig. 7 indicated that ~15% of the total radiolabeled P protein was found in the region of the gradient corresponding to ~8 to 10S (not shown). These data indicate that the SV5 P and L proteins can be isolated as a stable ~8 to 10S complex from virus-infected cell extracts, similar to the results obtained for cells cotransfected with P and L cDNAs.

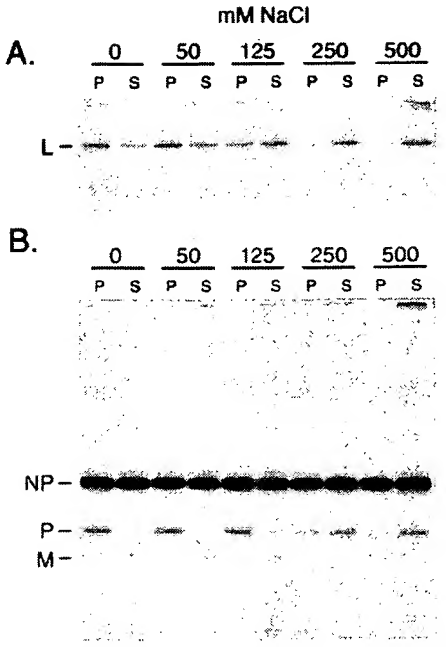

FIG. 6. Salt-dependent fractionation of L and P proteins from SV5-infected cells. HeLa T4 cells infected with SV5 (multiplicity of infection of ≈5) were radiolabeled at 19 hpi with Tran[^{35}S]label for 30 min. Cell extracts were prepared in 0.5 ml of NP-40-containing buffer that had been supplemented with NaCl to the indicated final salt concentrations. Following centrifugation (160,000 × *g*, 10 min), aliquots corresponding to equal amounts of the resulting pellet (P) and supernatant (S) fractions were boiled in SDS, diluted with buffer, and immunoprecipitated with 12 μl of either the anti-L protein serum (A) or the antiserum to SV5 viral proteins (B) before analysis by SDS-PAGE.

DISCUSSION

The members of the family of nonsegmented negative-sense RNA viruses, including the paramyxoviruses, rhabdoviruses, and filoviruses, are similar in genome structure and replication strategy (1, 13, 27). A common theme in the replication of

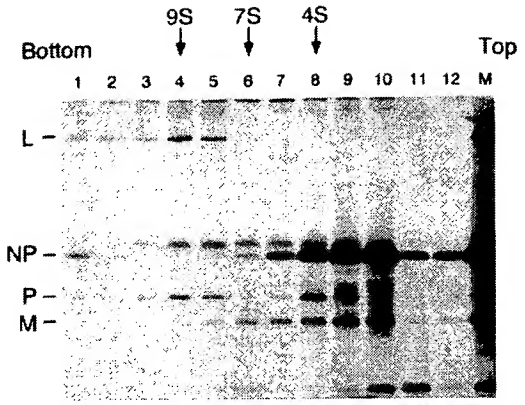

FIG. 7. Glycerol gradient sedimentation analysis of SV5-infected cell polypeptides. SV5-infected HeLa T4 cells were radiolabeled for 30 min at 15 hpi with Tran[^{35}S]label, and lysates were prepared in 0.5 ml of NP-40-containing buffer supplemented with 250 mM NaCl. Clarified lysate was analyzed by sedimentation through a 12-ml linear 5 to 20% glycerol gradient as described in Materials and Methods. Fractions (1 ml) were collected from the bottom of the gradient, and an aliquot of each fraction was adjusted to 0.5% SDS, boiled, diluted with buffer, and immunoprecipitated with a mixture of anti-L protein and anti-SV5 serum before analysis by SDS-PAGE. Arrows indicate the positions of 9S, 7S, and 4S sedimentation markers that were analyzed in a parallel gradient. M is a marker lane of proteins immunoprecipitated from the cell lysate prior to loading onto the gradient.

these viruses is the use of a large multifunctional L polypeptide to catalyze many of the steps in RNA synthesis and processing. However, the L protein does not carry out these functions alone, as viral RNA synthesis requires a second polymerase-associated viral protein P (or VP35 for the filoviruses [27]). Indirect evidence from in vitro reconstitution experiments (10, 15), as well as results obtained by using biochemical and immunoelectron microscopic techniques, have indicated that the L and P polypeptides physically interact to form an active polymerase complex (18, 28, 36).

The goal of the experiments reported here was to use cDNA-derived L and P to identify regions of the paramyxovirus L protein that are important for interaction with P. The interaction of L with P was not disrupted by large deletions within the C-terminal portion of the L molecule, as determined by two independent assays. In contrast, all of the deletion mutants that were altered in the N-terminal half of L were defective in the formation of stable complexes with P. Surprisingly, L mutants that lacked only the N-terminal 100 amino acids (~5% of the full L protein; mutant 1) or contained small N-terminal deletions that individually removed conserved domains I to III (mutants 14 to 16) were not detected as ~8 to 10S L-P complexes on glycerol gradients. The defect in the ability of these N-terminal deletion mutants to interact with P could reflect the fact that these alterations have induced a global malfolding of L or that the P-binding site has been masked rather than deleted. A more interesting possibility is that the N-terminal site for P binding is composed of widely dispersed discontinuous regions in L that fold into a secondary structure easily disrupted by deletions.

It has been proposed on a theoretical basis alone that the viral L protein contains distinct domains that may be responsible for the individual steps in RNA synthesis (34, 39). The data presented here provide the first experimental evidence in support of a domain structure for L. These results are consistent with a proposal that the structure of the L polypeptide consists of two independently folding halves; the N-terminal half of L contains the sites for P binding and the region which is thought to be the active site for RNA polymerization (34, 40), whereas the C-terminal half is not required for P binding but may be involved in interactions with the nucleocapsid. Alternatively, the C-terminal half of L could contain auxiliary functions not directly involved in RNA polymerization, such as the sites which catalyze the methylation or guanylation of mRNA (17). The deletions that created L protein mutants 7 and 8 removed large segments from the C-terminal half of L, and in both cases this included a precise deletion of conserved domain V (Fig. 1). All mutations that extended into L sequences located N-terminal to domain V resulted in polypeptides defective in P binding (e.g., mutants 10 to 13), suggesting that conserved region V may represent a border between two independently folding halves of L. Domain V is rich in cysteine (Cys) and histidine (His) residues that are conserved both in sequence and in spacing among viral L proteins (34, 39). A comparison of L protein sequences has led to the proposal that this Cys- and His-rich region may represent either a site for metal binding or a region that stabilizes the L structure (27, 34). In support of a possible role of this domain in L protein stability, it has been reported recently that amino acid changes associated with a transcription thermosensitivity phenotype for the VSV mutant *ts*G16(I) map to a Cys residue in conserved domain V of the L protein (19). Thus, the results from the deletion mutagenesis described here can be interpreted in the context of previous data to suggest a simple model for the structure of the L protein in which the Cys- and His- rich conserved domain V divides the molecule into two independently folding domains.

Although untested, it is very unlikely that the SV5 L protein deletion mutants described here would be active in RNA synthesis. Nevertheless, these results have important implications for future mutagenesis of other L proteins which might be targeted to disrupt an enzymatic activity without destroying the prerequisite interactions of L with the P protein. As a recent example, Canter et al. (5) have described a transcription-defective VSV L protein mutant that was constructed by deletion of a small C-terminal conserved region (domain VI). However, because of lack of prior knowledge of the location of the P-binding site on the VSV L protein and the ability of this altered L to bind to P, no conclusion concerning the exact role of this domain of L in RNA synthesis could be reached. By extension of the conclusions reached here to the structure of the VSV L protein, one would predict that the mutant VSV L protein containing this C-terminal deletion (5) would still bind P and that this C-terminal mutation has altered L activity by a different means.

The stoichiometry of the L and P polypeptides in the ~8 to 10S SV5 L-P complex is not presently known. The analysis of the ratio of these proteins is complicated by the fact that both free L and L-P sediment to the same region of the glycerol gradients, presumably as a result of the small contribution of P (M_r ~ 46,000) to the size of the L-P complex. Likewise, the ratio of L to P in the active polymerase complex that is bound to the NC template unknown. However, after purification from virions by ion-exchange column chromatography, the VSV polymerase complex is reported to be an L_1P_1 heterodimer that sediments on glycerol gradients as a ~11S particle (28). The similarity in the SV5 ~8 to 10S L-P sedimentation value reported here to that of the ~11S VSV L-P particle (28) suggests that the SV5 L-P complex may also exist as a heterodimer.

At the present time, it is unclear why only a small portion of

the cDNA-derived soluble P protein is found as the ~8 to 10S species. Titration experiments (not shown) indicated that the relative amount of P in the ~8 to 10S fraction did not change dramatically with large increases in the amount of either P or L plasmid that was transfected, suggesting that L-P assembly may be governed by additional factors other than concentration. In lysates from SV5-infected cells, ~15% of the pulse-radiolabeled P protein that had been solubilized was found in the ~8 to 10S complex, while the remainder was found in the same gradient fractions as the NP protein. These two forms of P may correspond to the P-L and P-NP complexes, respectively, which have been previously described by using biochemical approaches (18) and by immunoelectron microscopy (36). Alternatively, the sedimentation of cDNA- or SV5-derived P as two separate populations may be the result of either an instability of the complex, an equilibrium between the L-bound and unbound forms of P, or two distinct pools of P protein which differ from each other in some manner. In support of this latter possibility, distinct subpopulations of the VSV P protein which differ in phosphorylation and transcription activity have been described (3, 8). This modification to the P polypeptide may control or be the result of interactions with the L polypeptide. Experiments are in progress to determine if the two forms of the SV5 P reported here differ by their phosphorylation patterns and to determine the role of phosphorylation in the assembly of the paramyxovirus L-P complex.

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Evaluation of attenuation, immunogenicity and efficacy of a bovine parainfluenza virus type 3 (PIV-3) vaccine and a recombinant chimeric bovine/human PIV-3 vaccine vector in rhesus monkeys

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Restricted replication in the respiratory tract of rhesus monkeys is an intrinsic property of bovine parainfluenza virus type 3 (bPIV-3) strains. This host range phenotype of bPIV-3 has been utilized as a marker to evaluate the attenuation of bPIV-3 vaccines for human use. Two safety, immunogenicity and efficacy studies in primates evaluated and compared three human parainfluenza virus type 3 (hPIV-3) vaccine candidates: biologically derived bPIV-3, a plasmid-derived bPIV-3 (r-bPIV-3) and a chimeric bovine/human PIV-3 (b/hPIV-3). These studies also examined the feasibility of substituting Vero cells, cultured in the presence or absence of foetal bovine serum, for foetal rhesus lung-2 (FRhL-2) cells as the tissue culture substrate for the production of bPIV-3 vaccine. The results demonstrated that (i) Vero cell-produced bPIV-3 was as attenuated, immunogenic and efficacious as bPIV-3 vaccine grown in FRhL-2 cells, (ii) plasmid-derived bPIV-3 was as attenuated, immunogenic and efficacious as the biologically derived bPIV-3 and (iii) the b/hPIV-3 chimera displayed an intermediate attenuation phenotype and protected animals completely from hPIV-3 challenge. These results support the use of bPIV-3 vaccines propagated in Vero cells in human clinical trials and the use of b/hPIV-3 as a virus vaccine vector to express foreign viral antigens.

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INTRODUCTION

Human parainfluenza virus type 3 (hPIV-3), a causative agent of lower respiratory tract diseases, such as virus pneumonia and bronchiolitis in infants and children, is closely related to bovine parainfluenza virus type 3 (bPIV-3) (Welliver *et al.*, 1986). Both are members of the family *Paramyxoviridae*. There is currently no licensed vaccine for hPIV-3. The amino acid sequences of the haemagglutinin–neuraminidase (HN) and fusion (F) proteins of bPIV-3 and hPIV-3 are greater than 75% related (Bailley *et al.*, 2000), the bovine and human viruses are approximately 25% antigenically related by cross-neutralization (van Wyke Coelingh *et al.*, 1988) and at least five neutralization epitopes on the HN and F proteins are shared between the two viruses (Coelingh *et al.*, 1986; Klippmark *et al.*, 1990). In addition, the major viral non-glycoproteins N, M and L are greater than 85% related between bPIV-3 and hPIV-3. Neutralizing antibodies to HN and F play a central role in resistance to PIV-3 infection and illness in humans, and T-cell response to these may be important for resolution of infection. The high degree of relatedness between the two viruses provided the rationale of using bPIV-3 as a live virus vaccine to stimulate both humoral and cellular immunity capable of protecting humans from hPIV-3 disease. Another live attenuated hPIV-3 vaccine candidate being considered for human clinical studies was generated by reverse genetics, yielding a recombinant bPIV-3 (r-bPIV-3). r-bPIV-3 is phenotypically identical to the biologically derived bPIV-3 (Haller *et al.*, 2000, 2001).

The bPIV-3 vaccine strain (bPIV-3/Kansas/15626/84) used here was isolated in 1984 from a calf with pneumonia in Kansas and was shown to be immunogenic in hamsters (Haller *et al.*, 2000) and in Old World and New World non-human primates (van Wyke Coelingh *et al.*, 1988). Compared to hPIV-3, bPIV-3 was approximately 100- to 1000-fold restricted for replication in the lower (LRT) and

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upper respiratory tract (URT) of rhesus monkeys. This host range restriction was correlated with reduced virulence in humans and a live bPIV-3 vaccine was shown to be safe, attenuated and immunogenic in children (Karron *et al.*, 1995, 1996). These results provided the basis for development of bPIV-3 as a vector to express foreign viral antigens. Initially, the bPIV-3 F and HN genes were substituted with those of hPIV-3 to generate a chimeric bovine/human PIV-3 (b/hPIV-3) vaccine candidate, with the goal of inducing an immune response that is antigenically homologous with respect to the hPIV-3 HN and F proteins, which are targets for neutralizing antibodies (Haller *et al.*, 2000, 2001). Since the attenuation determinants reside in multiple bPIV-3 genes and b/hPIV-3 contains 78% of the bPIV-3 genome, b/hPIV-3 is expected to retain the attenuation phenotype (Schmidt *et al.*, 2000).

A live attenuated bPIV-3 vaccine was evaluated in a phase 2 clinical trial (Lee *et al.*, 2001; Greenberg *et al.*, 1999) in which the vaccine was administered intranasally, concurrently with other routine vaccinations, to infants at 2, 4 and 6 months of age, followed by a booster immunization at 12–15 months of age. This trial demonstrated that bPIV-3 was safe and well tolerated in infants and that the immunization elicited a humoral antibody response to bPIV-3 that cross-reacted with hPIV-3. There were no significant differences between vaccine and placebo groups with respect to rates of fever $>101\,^{\circ}F$ or other post-vaccination adverse events (Greenberg *et al.*, 1999). Since the bPIV-3 vaccine used in this phase 2 trial was prepared on foetal rhesus lung-2 (FRhL-2) cells, it was necessary to identify cell substrates suitable for commercial production of bPIV-3, as routine vaccine manufacture using FRhL-2 cells was not feasible. After testing bPIV-3 replication in 10 cell lines, Vero cells were identified as the cell substrate for vaccine production based on vaccine yield considerations. Although no live virus vaccine manufactured in Vero cells has yet been approved by the Food and Drug Administration (FDA) for use in humans, the FDA has issued recommendations for producing vaccines in Vero cells.

Two primate studies were performed to characterize attenuation, immunogenicity and efficacy of potential PIV-3 vaccines following propagation in either FRhL-2 or Vero cells. Study A compared bPIV-3 vaccine manufactured in Vero cells [bPIV-3(V)] to that produced in FRhL-2 cells [bPIV-3(F)] with respect to antigenicity in serological assays as well as immunogenicity and attenuation in non-human primates. In study B, the feasibility of using Vero cells grown in medium without foetal bovine serum (FBS) for PIV-3 vaccine manufacture was evaluated to reduce the possibility of contracting bovine spongiform encephalopathy. Also in study B, attenuation, immunogenicity and efficacy of plasmid-derived r-bPIV-3 and b/hPIV-3 were studied to evaluate their potential application as vaccine vectors.

The results of the two primate studies showed that the attenuation, immunogenicity and efficacy profiles of bPIV-3(V) and bPIV-3(F) vaccines were similar and supported the use of Vero cells for vaccine manufacture. The growth of bPIV-3, r-bPIV-3 or b/hPIV-3 in serum-free (SF) Vero cells did not affect vaccine yield, virus replication, immunogenicity or efficacy in primates. The data presented here also showed that r-bPIV-3 and b/hPIV-3 were attenuated and efficacious in primates. They represent novel virus vectors and vaccine candidates to be evaluated in future clinical studies.

METHODS

Cells and viruses. Vero cells obtained from the WHO cell bank were maintained in DMEM supplemented with 2 mM L-glutamine, non-essential amino acids (NEAA) and 5% FBS. FRhL-2, Madin–Darby bovine kidney (MDBK) and rhesus monkey kidney (LLC-MK2) cells were cultured in DMEM containing 2 mM L-glutamine, NEAA, antibiotics and 10% FBS. All media components were purchased from HyClone Laboratories. SF Vero cells were grown in OptiPRO SFM medium (Invitrogen) supplemented with 4 mM L-glutamine and antibiotics.

bPIV-3 (Kansas/15626/84), hPIV-3 (JS) and hPIV-3 (Washington/47885/57) were propagated in Vero and FRhL-2 cells. Cells were infected with bPIV-3 or hPIV-3 at an m.o.i. of 0·001 $TCID_{50}$ per cell and incubated at 37 °C. Culture medium was centrifuged at 600 *g* for 10 min. The supernatant was stabilized by adding 10× SPG (2·18 M Sucrose, 0·038 M KH_2PO_4, 0·072 M K_2HPO_4 and 0·054 M L-glutamate) to a final concentration of 1×. bPIV-3, hPIV-3, r-bPIV-3 and b/hPIV-3 were grown in SF Vero cells. Briefly, Vero cells were infected at an m.o.i. of 0·01 $TCID_{50}$ per cell and overlaid with Opti-MEM I. At 3 days post-infection, cells and supernatants were collected, SPG was added and virus stocks were stored at −70 °C. Virus titres were determined by $TCID_{50}$ assays.

Rhesus monkey studies

Study A. Rhesus monkeys (1–4 years old; 3–5 kg) were pre-screened for PIV-3 antibodies by haemagglutination inhibition assay (HAI) and seronegative animals were randomly assigned to five groups (four monkeys per group). Groups of monkeys were anaesthetized with a ketamine/valium mixture and vaccinated intratracheally and intranasally via drops with bPIV-3(F) vaccine, bPIV-3(V) vaccine, hPIV-3(F) or hPIV-3(V). On day 0, each animal received 2×10^5 $TCID_{50}$ of virus or placebo (Liebovitz-15 medium) in a 2 ml volume. The nasal dose volume was 0·5 ml per nostril and the intratracheal dose volume was 1 ml. On day 28 post-vaccination, each animal received a second dose that was identical to the first dose. On day 56 post-vaccination, the animals were challenged intratracheally and intranasally with 2×10^5 $TCID_{50}$ hPIV-3/JS (FRhL-2 cells). Nasopharyngeal (NP) swabs were taken daily for 12 days following administration of dose 1, dose 2 and challenge. Tracheal lavage (TL) specimens were collected on days 1, 3, 5, 7 and 10 following each dose of the vaccine as well as the challenge. Blood samples were obtained from the femoral vein on days 0, 28, 56 and 84.

Study B. PIV-3-seronegative rhesus monkeys (1–4 years old; 3–5 kg) were assigned to four groups (four monkeys per group). Monkeys were immunized intranasally and intratracheally, as in study A, with r-bPIV-3, b/hPIV-3 and hPIV-3/JS. On day 0, each animal received a dose of 2 ml containing 1×10^5 $TCID_{50}$ of virus. The placebo animal group received the same dose volume of

Opti-MEM. On day 28, all animals were challenged intratracheally and intranasally with 2×10^5 $TCID_{50}$ hPIV-3/JS (Vero cells). NP swabs were collected daily for 11 days and TL specimens were collected on days 1, 3, 5, 7 and 9 post-immunization and post-challenge. Blood samples were collected on days 0, 7, 14, 21, 28, 35, 42, 49 and 56.

Quantification of virus shedding. bPIV-3 and hPIV-3 present in the primate NP and TL specimens were quantified by $TCID_{50}$ assays using MDBK, LLC-MK2 or Vero cells. Previous studies had indicated that MDBK cells were sensitive for bPIV-3 replication and LLC-MK2 cells were sensitive for hPIV-3 replication. To confirm that the CPE observed was produced by virus infection and not mucus and cell debris present in the NP and TL samples, the $TCID_{50}$ assay was modified to include haemadsorption with guinea pig (gp) erythrocytes. Haemadsorption was recorded visually and virus titres were calculated according to the Karber modification of the Reed–Muench equation (Reed & Muench, 1938). Mean peak virus titres represent the mean of the peak $TCID_{50}$ titre measured on any of the 12 days following dose 1, dose 2 or challenge.

HAI assay. HAI assays were performed by incubating serial 2-fold dilutions of monkey serum at 25 °C for 30 min with 8 HA units per 0·05 ml of either bPIV-3 or hPIV-3. Subsequently, gp red blood cells were added to each well, incubation was continued for 90 min and each well was then observed for haemagglutination. HAI titres were expressed as the reciprocal of the highest dilution of antiserum that inhibited virus-mediated agglutination of erythrocytes. To facilitate calculation of the geometric mean titre (GMT), HAI titres of $\leqslant 1:4$ (the lowest serum dilution tested) were assigned a titre of 1 : 2.

Microneutralization assays. Microneutralization assays were performed on MDBK, LLC-MK2 or Vero cells. Serial 2-fold dilutions of serum, starting at 1 : 4, were incubated at 37 °C for 60 min with 100 $TCID_{50}$ of either bPIV-3 or hPIV-3. Then, virus/serum mixtures were transferred to cell monolayers in 96-well plates and incubated at 37 °C for 6 days, after which all wells were observed for CPE. Neutralization titres were expressed as the reciprocal of the highest serum dilution that inhibited CPE. Neutralization antibody titres of $\leqslant 1:4$ (the lowest serum dilution tested) were assigned a titre of 1 : 2.

PIV-3 IgA ELISA. Glycoprotein extracts of bPIV-3/Kansas/15626/84 and hPIV-3/Washington/47885/57 were prepared as follows. bPIV-3- or hPIV-3-infected MDBK cell supernatants were centrifuged at 100 000 *g* to pellet the virus, which was then resuspended in PBS, overlaid on a 30 % sucrose cushion and centrifuged at 100 000 *g* for 90 min. The pellet was resuspended in PBS, treated with an equal volume of 1 % Nonidet P-40 (in PBS) for 4 h at room temperature and centrifuged at 100 000 *g* for 90 min. The extracted HN glycoprotein supernatant was stored frozen. Serial 4-fold dilutions of serum were added to 96-well plates that had been coated previously with glycoprotein extracts of bPIV-3 or hPIV-3. Bound monkey IgA was detected by the addition of horseradish peroxidase-conjugated sheep anti-human IgA antiserum, followed by 3,3′,5,5′-tetramethylbenzidine substrate. The absorbance of the substrate was read spectrophotometrically at 450 nm. The absorbance values obtained with rhesus monkey serum samples were compared to a standard curve of a human reference IgA antiserum. The amount of bound serum IgA was expressed as units of IgA ml^{-1}. Test serum samples that produced a signal in the IgA ELISA that was $\leqslant 7{\cdot}8$ units IgA ml^{-1} (the lowest value represented in the standard curve of the reference antiserum) was assigned a titre of 7·8 units IgA ml^{-1}.

RESULTS

Attenuation and immunogenicity in rhesus monkeys after a single dose of bPIV-3 manufactured in Vero or FRhL-2 cells (Study A)

Vaccine safety and efficacy were evaluated in a rhesus monkey replication model of attenuation. To determine whether the attenuation phenotype associated with bPIV-3 would be preserved in vaccines produced in Vero as well as FRhL-2 cells, virus shedding after the first immunization (dose 1) was measured and replication of bPIV-3(F) vaccine was compared to that of hPIV-3(F), and replication of bPIV-3(V) vaccine was compared to hPIV-3(V).

Following primary vaccination with bPIV-3(F), monkeys shed for 9 days in the nasopharynx, displaying a mean peak titre of $10^{3{\cdot}1}$ $TCID_{50}$ ml^{-1}, and for 8 days in the trachea, with mean peak titres of $10^{2{\cdot}6}$ $TCID_{50}$ ml^{-1}. In contrast, vaccination with non-attenuated hPIV-3(F) resulted in virus shedding for 10 days in the nasopharynx, showing mean peak titres of $10^{4{\cdot}5}$ $TCID_{50}$ ml^{-1}, and for 9 days in the trachea, with peak titres of $10^{3{\cdot}8}$ $TCID_{50}$ ml^{-1} (Table 1). Thus, shedding of bPIV-3(F) was 25-fold lower in the nasopharynx and 16-fold lower in the trachea compared to hPIV-3(F). Monkeys that received bPIV-3(V) shed $10^{3{\cdot}3}$ $TCID_{50}$ ml^{-1} for 9 days in the nasopharynx and $10^{3{\cdot}4}$ $TCID_{50}$ ml^{-1} for 8 days in the trachea. In contrast, monkeys vaccinated with hPIV-3(V) shed $10^{5{\cdot}1}$ $TCID_{50}$ ml^{-1} for 9 days in the nasopharynx and $10^{5{\cdot}3}$ $TCID_{50}$ ml^{-1} for 7 days in the trachea (Table 1). The shedding of bPIV-3(V) was 63-fold lower in the nasopharynx and 79-fold lower in the trachea compared to hPIV-3(V).

The antibody responses induced in the animals upon immunization were studied by HAI, neutralization and IgA ELISA assays to determine whether the cell line used for manufacture had an influence on immunogenicity. All monkeys mounted a serum HAI antibody response to bPIV-3 (a 'homologous' antibody response) following the initial vaccination with either bPIV-3(F) or bPIV-3(V) (Table 2). Vaccination with bPIV-3(F) and bPIV-3(V) resulted in a homologous HAI GMT of 90·5 and 64·0, respectively. All vaccinated monkeys also mounted a HAI antibody response to hPIV-3 (a 'heterologous' antibody response). Vaccination with bPIV-3(F) and bPIV-3(V) resulted in a heterologous HAI GMT of 8·0 and 16·0, respectively (Table 2). Titres within 4-fold of each other in this serological assay are considered comparable.

The primary serum neutralizing antibody response was similar for both bPIV-3 vaccines tested (Table 3). bPIV-3(F) and bPIV-3(V) induced a homologous neutralizing antibody GMT of 51·6 and 70·8, respectively, in a neutralization assay that utilized bPIV-3 grown on FRhL-2 cells. The homologous GMT measured was also comparable for the two vaccines when the assay was performed using bPIV-3 prepared in Vero cells. Consistent with the serum HAI results, bPIV-3(F) and bPIV-3(V) induced a heterologous

Table 1. Comparison of replication and efficacy of bPIV-3, hPIV-3, r-bPIV-3 and b/hPIV-3 in rhesus monkeys (Studies A and B)

Immunizing virus‡	No. of animals	Pre-challenge mean peak virus titre*		Post-challenge mean peak virus titre†	
		NP swab	TL	NP swab	TL
Study A					
bPIV-3/Kansas/15626/84(F)§	4	3·1±0·6	2·6±1·3	0	0
bPIV-3/Kansas/15626/84(V)§	4	3·3±0·6	3·4±0·7	0	1·0±0·0
hPIV-3/JS(F)	4	4·5±0·8	3·8±0·4	0	0
hPIV-3/Washington/47885/57(V)	4	5·1±1·1	5·3±0·7	0	0
Placebo	4	0	0	4·1±0·7	3·6±0·2
Study B					
r-bPIV-3/Kansas/15626/84	4	3·6±0·7	3·8±0·5	0·5±0·8	0·3±0·5
b/hPIV-3	4	3·9±0·7	4·9±0·8	0	0
hPIV-3/JS	4	5·0±0·6	5·7±0·7	1·2±1·9	0
Placebo	4	0	0	4·8±1·1	5·3±0·6

*Mean peak virus titre is expressed as $\log_{10}$ $TCID_{50}$ ml^{-1} ±SE and is the mean of the highest titre of virus of each animal in the specific group during the course of the study.

†Animals were challenged on day 28 (Study B) or day 56 (Study A) with 10^5 $TCID_{50}$ of wild-type hPIV-3/JS intranasally and intratracheally.

‡Animals were inoculated with 10^5 $TCID_{50}$ of the indicated virus intranasally and intratracheally. For study B, the viruses were propagated in SF Vero cells.

§F, Virus stocks grown in FRhL-2 cells; V, virus stocks grown in Vero cells.

neutralizing antibody GMT of 34·9 and 26·9, respectively, when the assay was performed with hPIV-3 grown on LLC-MK2 cells.

When the antibody response was measured using an IgA ELISA, the titres were similar (within 2-fold) for bPIV-3 vaccine produced in either FRhL-2 or Vero cells (Table 3). bPIV-3(F) and bPIV-3(V) induced a homologous IgA antibody GMT of 52·2 and 53·6, respectively, and a heterologous GMT of 10·0 and 20·1, respectively.

Immune response of rhesus monkeys to a bPIV-3 vaccine booster dose (Study A)

On day 28, all animals received booster vaccinations (dose 2), identical to the vaccines or viruses used for dose 1. bPIV-3(F) or bPIV-3(V) vaccines were administered to study whether the antibody response could be enhanced. bPIV-3 shedding was not detected except in one of four animals, which displayed low levels (10^1 $TCID_{50}$ ml^{-1}) of bPIV-3(V) shedding in the trachea. In contrast to the results with the bPIV-3 vaccine, hPIV-3(F) or hPIV-3(V) were not observed in either nasopharyngeal or tracheal specimens after dose 2 (data not shown).

The serum HAI GMT to bPIV-3 continued to increase after the booster dose of bPIV-3 vaccine produced in FRhL-2 or Vero cells. After re-vaccination with a second dose of bPIV-3(F) or bPIV-3(V), the homologous HAI GMT increased 1·4-fold (from 90·5 to 128·0 and from 64·0 to 90·5, respectively) (Table 2). Re-vaccination also resulted in a similar increase in the heterologous HAI GMT for both bPIV-3 vaccines tested (from 8·0 to 32·0 and from 16·0 to 22·6, respectively).

Table 2. Serum HAI antibody response upon immunization of rhesus monkeys with bPIV-3 (F or V) and hPIV-3 (F or V) (study A)

Immunizing virus	No. of animals	Serum HAI GMT							
		Day 0 (pre-dose)		Day 28 (post-dose 1)		Day 56 (post-dose 2)		Day 84 (post-challenge)	
		hPIV-3	bPIV-3	hPIV-3	bPIV-3	hPIV-3	bPIV-3	hPIV-3	bPIV-3
bPIV-3(F)	4	2·0	2·4	8·0	90·5	32·0	128·0	362·0	362·0
bPIV-3(V)	4	2·0	2·0	16·0	64·0	22·6	90·5	215·3	181·0
hPIV-3(F)	4	2·0	2·0	215·3	53·8	430·5	90·5	724·1	128·0
hPIV-3(V)	4	2·0	2·0	256·0	38·1	215·3	32·0	608·0	107·6
Placebo	4	2·0	2·0	2·0	2·0	2·0	2·4	256·0	53·8

Table 3. Serum neutralizing antibody and serum IgA ELISA responses upon immunization of rhesus monkeys with bPIV-3 (F or V) and hPIV-3 (F or V) (Study A)

Immunizing virus	No. of animals	GMT serum neutralizing antibody on day 28			GMT serum IgA antibody on day 28 (IgA units ml^{-1})	
		bPIV-3 (FRhL)*,†	bPIV-3 (Vero)*,†	hPIV-3 (LLC-MK2)*,†	bPIV-3 glycoproteins*,‡,§	hPIV-3 glycoproteins*,‡,§
bPIV-3(F)	4	51·6	173·5	34·9	52·2	10·0
bPIV-3(V)	4	70·8	221·7	26·9	53·6	20·1
hPIV-3(F)	4	51·6	119·1	32·0	115·3	223·5
hPIV-3(V)	4	39·2	135·8	29·3	80·8	137·0
Placebo	4	2·0	2·0	2·0	7·8	7·8

*Antigen used in the neutralization or ELISA assay. Day 0, pre-dose; day 28, post-dose 1.

†Serum neutralizing antibody titres on day 0 were 2·0.

‡bPIV-3 and hPIV-3 used to isolate the glycoproteins were propagated on MDBK cells.

§The lowest value on the IgA ELISA standard curve of the reference antiserum is 7·8 IgA units ml^{-1}. Serum IgA ELISA titres on day 0 were 7·8 IgA units ml^{-1}.

Efficacy of bPIV-3 vaccine in rhesus monkeys against hPIV-3 challenge (Study A)

It was important to demonstrate that bPIV-3 vaccines produced in either FRhL-2 or Vero cells were efficacious in rhesus monkeys and protected from hPIV-3 challenge. Indeed, the only samples showing challenge virus shedding were collected from the placebo group. Placebo recipients shed challenge virus for 10 days in the nasopharynx with mean peak titres of $10^{4.1}$ $TCID_{50}$ ml^{-1} and for 9 days in the trachea, where mean peak titres of $10^{3.6}$ $TCID_{50}$ ml^{-1} were observed. In contrast, virus was not shed post-challenge in the nasopharynx of monkeys previously vaccinated with two doses of bPIV-3(F) or bPIV-3(V) (Table 1). Similarly, hPIV-3 challenge virus was not detected in tracheal specimens from these vaccinated animals, with the exception of a single sample that contained the minimum detectable level of virus (10^1 $TCID_{50}$ ml^{-1}). Challenge hPIV-3 was also not observed in either nasopharyngeal or tracheal specimens obtained from animals that had received two inoculations of hPIV-3(V) or hPIV-3(F).

Following hPIV-3 challenge, the serum HAI GMT was boosted in animals that had been previously vaccinated with bPIV-3 vaccine produced in either FRhL-2 or Vero cells (Table 2). The GMT to bPIV-3 doubled in animals previously vaccinated with bPIV-3(F) or bPIV-3(V), and the GMT to hPIV-3 increased 10-fold in animals previously vaccinated with either bPIV-3(F) or bPIV-3(V).

Attenuation and immunogenicity of r-bPIV-3 and b/hPIV-3 in rhesus monkeys (Study B)

Development of bPIV-3 as a vaccine vector necessitated the construction of an infectious cDNA of the bPIV-3 genome such that foreign antigens could be introduced at the cDNA level (Haller *et al.*, 2000). We demonstrated that r-bPIV-3 was phenotypically identical to the biological bPIV-3 *in vitro* and *in vivo* using hamsters (Haller *et al.*, 2001). However, hamsters do not represent a sensitive model for studying bPIV-3 attenuation. To show that r-bPIV-3 and b/hPIV-3, containing the hPIV-3 F and HN genes, retained the attenuation determinants, a rhesus monkey study was performed. Restricted replication of bPIV-3 in the LRT and URT of rhesus monkeys when compared to hPIV-3 can be correlated and used as an attenuation marker of bPIV-3 in humans. All of the virus stocks used in this study for immunization of the animals were grown in SF Vero cells.

In this study, r-bPIV-3 replicated to mean peak titres of $10^{3.6}$ and $10^{3.8}$ $TCID_{50}$ ml^{-1} in the nasopharynx and trachea of immunized monkeys, respectively. These titres were comparable to those obtained with biological bPIV-3. Monkeys that received hPIV-3/JS shed $10^{5.0}$ and $10^{5.7}$ $TCID_{50}$ ml^{-1} in the URT and LRT. Thus, shedding of r-bPIV-3 was 25-fold lower in the nasopharynx and 79-fold lower in the trachea compared to hPIV-3. b/hPIV-3 demonstrated an intermediate attenuation phenotype compared to r-bPIV-3 and hPIV-3. b/hPIV-3 replication was reduced 13-fold in the nasopharynx and 6-fold in the trachea of rhesus monkeys compared to hPIV-3 (Table 1).

The sera from all vaccinated animals on day 28 post-immunization, with the exception of the placebo recipients, contained serum HAI and neutralizing antibodies (Tables 4 and 5). Vaccination of monkeys with r-bPIV-3 resulted in a HAI titre of 64·0 on day 21 when tested with bPIV-3 antigen (Table 4). When the same serum was tested with a heterologous hPIV-3 antigen, the HAI titre was 11·3. At 1 week later (day 28), the heterologous HAI titre was comparable to the day 21 titre (i.e. 13·5). r-bPIV-3 elicited a similar HAI antibody response to both homologous and heterologous antigens as biological bPIV-3 (Tables 2 and 4). Interestingly, the HAI titres from day 21 sera of animals

Table 4. Serum HAI antibody response upon immunization of rhesus monkeys with r-bPIV-3, b/hPIV-3 and hPIV-3 (Study B)

Immunizing virus	No. of animals	Serum HAI GMT*							
		Day 0 (pre-dose)		Day 21 (post-dose)		Day 28 (pre-challenge)		Day 56 (post-challenge)	
		hPIV-3†	bPIV-3†	hPIV-3	bPIV-3	hPIV-3	bPIV-3	hPIV-3	bPIV-3
r-bPIV-3	4	2·0	2·0	11·3	64·0	13·5	45·3	256·0	181·0
b/hPIV-3	4	2·0	2·0	76·1	11·3	53·8	6·7	304·4	26·9
hPIV-3/JS	4	2·0	2·0	215·3	22·6	107·6	16·0	215·3	32·0
Placebo	4	2·0	2·0	2·0	2·0	2·0	2·0	608·9	76·1

*Day 0, pre-dose; day 21, 3 weeks post-dose; day 28, pre-challenge; day 56, 4 weeks post-challenge.
†Antigen used in the HAI assay.

immunized with b/hPIV-3 were 76·1 for the hPIV-3 antigen and 11·3 for the bPIV-3 antigen. The HAI titres for hPIV-3 were 215·5 for the homologous hPIV-3 antigen and 22·6 when using bPIV-3 antigen (Table 4). The neutralizing antibody response induced by r-bPIV-3 and b/hPIV-3 on day 28 was measured on both bPIV-3 and hPIV-3 substrates. r-bPIV-3 induced a neutralizing antibody titre of 107·6 and 9·5 for bPIV-3 and hPIV-3 antigens, respectively. These titres were similar to those observed for biological bPIV-3. b/hPIV-3 elicited GMTs of 26·9 and 90·5 for bPIV-3 and hPIV-3 substrates, respectively. hPIV-3 displayed neutralizing antibody titres of 38·1 for the bPIV-3 substrate and 304·4 for the hPIV-3 antigen (Table 5).

r-bPIV-3- or b/hPIV-3-immunized rhesus monkeys were protected from hPIV-3 challenge (Study B)

To demonstrate that r-bPIV-3 and b/hPIV-3 were efficacious, an hPIV-3 challenge was carried out in rhesus monkeys. In contrast to Study A, in Study B only a single vaccine dose was administered to the animals prior to hPIV-3 challenge. Placebo recipients challenged with hPIV-3 shed $10^{4·8}$ and $10^{5·3}$ $TCID_{50}$ ml^{-1} in the nasopharynx and trachea, respectively (Table 1). The animals that had received r-bPIV-3, b/hPIV-3 or hPIV-3 displayed greatly reduced peak titres in both the LRT and URT (Table 1). For r-bPIV-3, $10^{0·5}$ and $10^{0·3}$ $TCID_{50}$ hPIV-3 ml^{-1} was observed post-challenge. Interestingly, shed challenge virus was not detected in samples derived from b/hPIV-3-vaccinated primates, even after haemadsorption of the infected monolayers with gp red blood cells. Monkeys that were immunized with hPIV-3/JS shed $10^{1·2}$ $TCID_{50}$ ml^{-1} of challenge virus in the nasopharynx. The titre observed in the nasopharynx was due primarily to a single animal in that group that did not shed high titres of virus or develop a robust immune response following primary immunization.

Following hPIV-3 challenge, the serum HAI titres were boosted in all animals, independent of the vaccine received on day 0 (Table 4). The monkeys that were vaccinated with r-bPIV-3 increased HAI titres to both homologous bPIV-3 and heterologous hPIV-3 antigens 3-fold and 23-fold, respectively, over the titres observed on day 21. The animals immunized with b/hPIV-3 increased HAI titres for the hPIV-3 antigen 4-fold and to the bPIV-3 antigen 2-fold. The hPIV-3-vaccinated animals also increased the hPIV-3 HAI titre more so than for the bPIV-3 antigen. The neutralizing antibody response was also stimulated

Table 5. Serum neutralizing antibody response upon immunization of rhesus monkeys with r-bPIV-3, b/hPIV-3 and hPIV-3 (Study B)

Immunizing virus	No. of animals	Serum neutralizing GMT					
		bPIV-3 (grown in Vero cells)*			hPIV-3 (grown in LLC-MK2 cells)*		
		Day 0†	Day 28†	Day 56†	Day 0	Day 28	Day 56
r-bPIV-3	4	2·0	107·6	215·3	2·0	9·5	107·6
b/hPIV-3	4	2·0	26·9	45·3	2·0	90·5	215·3
hPIV-3/JS	4	2·0	38·1	32·0	2·0	304·4	107·6
Placebo	4	2·0	2·0	76·1	2·0	2·0	304·4

*Antigen used in the neutralization assay.
†Day 0, pre-dose; day 28, pre-challenge; day 56, 4 weeks post-challenge.

post-challenge (Table 5). Serum obtained on day 56 from monkeys vaccinated with r-bPIV-3 had a 2-fold increase in neutralizing antibody titres for the bPIV-3 antigen, and a 11-fold increase was observed for the hPIV-3 antigen. The animals that received b/hPIV-3 displayed only a slight increase in neutralizing antibody titres for day 56 serum when the bPIV-3 antigen was used. However, a 2-fold increase was observed with the hPIV-3 antigen. In contrast, hPIV-3-immunized animals showed a decrease in both bPIV-3 and hPIV-3 neutralizing antibodies.

DISCUSSION

The goal of the two primate studies described here was to demonstrate comparability of bPIV-3 vaccine manufactured in FRhL-2 and Vero cells with respect to attenuation, immunogenicity and efficacy, and to show that the plasmid-derived bPIV-3 and the chimeric b/hPIV-3 maintained the attenuation phenotype and protected primates from hPIV-3 challenge. The observation that the bPIV-3 vaccine was attenuated in rhesus monkeys and humans suggested that bPIV-3 could be used as a hPIV-3 vaccine or vector for delivery of foreign antigens (van Wyke Coelingh *et al.*, 1988; Karron *et al.*, 1995). This concept was supported by data obtained from clinical trials showing that a humoral immune response to hPIV-3 was elicited in children upon bPIV-3 vaccination (Karron *et al.*, 1995; Lee *et al.*, 2001). While the magnitude of the antibody responses in children following bPIV-3 vaccination suggested that they would be protected from hPIV-3, there are no data to support this conclusion. Therefore, we sought to demonstrate in a primate challenge model that vaccination with bPIV-3, r-bPIV-3 or b/hPIV-3 could protect animals from challenge with hPIV-3. Comparison of the results obtained in Study A and Study B demonstrated that r-bPIV-3 grown in SF Vero cells was as attenuated and efficacious as biological bPIV-3 produced in FRhL-2 or Vero cells cultured with FBS. Thus, FBS in the culture medium did not affect the immunogenicity of the various PIV-3 vaccines.

Two vaccines, bPIV-3(F) and bPIV-3(V), were compared directly for replication in the respiratory tract of rhesus monkeys (Study A). Virus shedding data obtained after the first dose of bPIV-3(F) were similar to results reported previously (van Wyke Coelingh *et al.*, 1988) and confirmed that this vaccine was attenuated compared to hPIV-3. Similarly, virus shedding results obtained after the first dose of bPIV-3(V) demonstrated that the vaccine was attenuated relative to hPIV-3. A quantitative analysis of the virus shedding results described above demonstrated that bPIV-3 vaccines, regardless of whether produced in FRhL-2 or Vero cells, were attenuated in the URT and LRT of rhesus monkeys in comparison to hPIV-3. Study B showed that r-bPIV-3, derived from cDNA, was attenuated compared to hPIV-3 and was as attenuated as biological bPIV-3 in the respiratory tract of rhesus monkeys. Both recombinant and biological bPIV-3 showed titre reductions of 25- and 63-fold in the URT, respectively, and 79-fold reductions in the LRT. b/hPIV-3 displayed an intermediate attenuation phenotype between the attenuated bPIV-3 and the non-attenuated hPIV-3, which indicated that some attenuation determinants are located in the F and HN genes, as suggested previously (Schmidt *et al.*, 2000). Whether this decrease of attenuation of b/hPIV-3 in rhesus monkeys will affect its safety profile in humans and whether insertion of additional viral genes encoding protective antigens from other respiratory viruses will compensate for the decreased attenuation of this vector remains to be determined.

Serological data obtained after the first dose confirmed that the bPIV-3 vaccines prepared in FRhL-2 and Vero cells were antigenically comparable and that immunogenicity was independent of the presence or absence of FBS in the Vero cell medium. The serological responses obtained from r-bPIV-3 were also similar to those of biological bPIV-3. All three serological parameters examined (HAI response, neutralizing response and IgA) showed that the humoral immune responses to the homologous bPIV-3 as well as to the heterologous hPIV-3 were within 2-fold of each other. Titres within 4-fold of each other in this type of serological assays are considered comparable. Interestingly, b/hPIV-3 elicited an immune response that resembled hPIV-3 more closely than bPIV-3. Humanizing the surface glycoproteins of bPIV-3 may have created a virus that elicits an immune response very similar to hPIV-3 infections.

In Study A, virus shedding was greatly reduced after administration of the second dose. While no shedding was observed in animals re-vaccinated with hPIV-3(V), hPIV-3(F) or bPIV-3(F), in one animal re-vaccinated with bPIV-3(V), a low level of virus shedding was observed. This suggested that even one dose of bPIV-3 vaccine can induce an immune response that can prevent, or significantly reduce, a secondary infection. This hypothesis was tested in Study B, in which the animals were challenged after receiving only a single vaccination dose. Here, the animals were protected effectively from hPIV-3 challenge. Animals that were vaccinated with r-bPIV-3 or b/hPIV-3 displayed no or low levels of virus shedding, similar to hPIV-3-vaccinated animals. The similarity in the virus shedding data obtained after the second dose with bPIV-3 vaccine grown in either Vero or FRhL-2 cells also demonstrated that the vaccines produced in the two cell substrates are equivalent. This observation was supported by the serological data that showed that while the serum HAI response to both homologous and heterologous viruses increased after the second dose of the two vaccines, the titres evoked were not different.

Both bPIV-3(F) and bPIV-3(V) vaccines provided complete protection from hPIV-3 in the URT and nearly complete protection in the LRT. The serological data showed that following the hPIV-3 challenge, a 2-fold increase in the HAI response against bPIV-3 antigen was induced in animals immunized with bPIV-3(F) or bPIV-3(V) and against hPIV-3 antigen in animals immunized with hPIV-3(F) or hPIV-3(V). In contrast, animals immunized with

bPIV-3(F) or bPIV-3(V) showed a 10-fold increase in HAI titre against hPIV-3 antigen after the animals were challenged with hPIV-3. This indicated that the bPIV-3(F) and bPIV-3(V) vaccines were capable of priming the immune system in rhesus monkeys so that the humoral response could be efficiently boosted when the animals encounter hPIV-3 antigen at a later time. This study focused on induction of serum HAI and neutralizing antibodies because of their established central role in protection from PIV-3 infection and disease in humans. Although homologous antibody titres were higher than heterologous titres, the high level of heterologous protection observed in this study is not unexpected because of the multiple HN and F neutralization epitopes that are shared by bPIV-3 and hPIV-3. The epitopes responsible for the generation of T-cell responses by the HN, F, and other 'internal' proteins, which are highly conserved between the bovine and human viruses, are currently unknown. However, based on the importance of cell-mediated immunity for humans to recover from paramyxovirus infections, it is likely that T-cell responses to bPIV-3 also contributed to the high level of heterologous protection observed after hPIV-3 challenge.

The primate studies described here showed that rhesus monkeys can be used as a challenge model to demonstrate efficacy of PIV-3 vaccines. While we cannot be certain that these vaccines will be efficacious in humans, previous studies have demonstrated a good correlation between the efficacy of bPIV-3 vaccines in monkeys and humans (van Wyke Coelingh *et al.*, 1988; Karron *et al.*, 1995, 1996) and none of the data obtained in this study would predict that these vaccines do not protect humans. The monkey model was employed here to show several points: (i) bPIV-3 vaccines prepared in Vero cells are equivalent to bPIV-3 vaccines grown in FRhL-2 cells. However, manufacturing bPIV-3 vaccines in Vero cells offers several commercial advantages over preparing bPIV-3 in FRhL-2 cells. For example, Vero cells grow faster than FRhL-2 cells and can be cultured for a greater number of passages. The peak titre of bPIV-3 is 30- to 50-fold higher in Vero than in FRhL-2 cells and is reached earlier. The combination of the shorter cycle time and higher titre render bPIV-3 vaccines derived from Vero cells preferable from a commercial standpoint; (ii) a recombinant bPIV-3 produced in SF Vero cells is as attenuated and efficacious as biological bPIV-3 produced in Vero cells grown in media containing FBS; (iii) b/hPIV-3 appears to display an intermediate attenuation phenotype between bPIV-3 and hPIV-3, protected completely from hPIV-3 challenge and stimulated high hPIV-3 antibody titres. Some genetic determinants responsible for the attenuation phenotype appear to be located in the bPIV-3 F and HN genes, which will be confirmed by performing additional primate studies. Nevertheless, b/hPIV-3 represents a promising vaccine vector to express antigens derived from other virus pathogens because additional gene insertions into the b/hPIV-3 genome are expected to attenuate virus replication in primates and humans. Thus, a balance should be achieved between virus replication, antigen expression and induction of a protective immune response in vaccinees.

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The future of respiratory syncytial virus vaccine development

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Background. **Respiratory syncytial virus (RSV) is the leading cause of viral lower respiratory tract illness in infants and children and is an important cause of lower respiratory tract illness in other populations. Despite decades of research there are currently no licensed vaccines for prevention of RSV disease.**

Methods. **A review of the obstacles to RSV vaccine development; current live, attenuated and subunit RSV vaccines in clinical development; and the potential for developing additional vaccine candidates based on recombinant technology.**

Results. **A number of biologically derived live attenuated RSV vaccines were evaluated in Phase I clinical trials in adults and children, and one vaccine (*cpts* 248/404) was evaluated in infants as young as 1 month of age. These vaccines displayed a spectrum of attenuation, with *cpts* 248/955 being the least attenuated and *cpts* 248/404 being the most attenuated candidate vaccine. None of these was sufficiently attenuated for young infants. The ability to generate recombinant RSV vaccines has led to the development of large numbers of candidate vaccines containing combinations of known attenuating point mutations and deletions of nonessential genes. Clinical evaluation of many of these candidates is in progress.**

Three types of RSV subunit vaccines have recently been evaluated in clinical trials: purified F glycoprotein vaccines (PFP-1, PFP-2 and PFP-3), BBG2Na and copurified F, G and M proteins. Additional studies of the F/G/M protein vaccine are being conducted.

Conclusions. **During the past 10 years, considerable progress has been made in RSV vaccine development. It is likely that different RSV vaccines will be needed for the various populations at risk.**

INTRODUCTION

Respiratory syncytial virus (RSV) is the most important cause of viral lower respiratory tract illness (LRI) in infants and children worldwide.[1,2] In the United States it is estimated that ~70 000 to 126 000 infants are hospitalized annually with RSV pneumonia or bronchiolitis and that the rate of hospitalization for bronchiolitis has increased since 1980.[3] Although originally described as a pediatric pathogen, RSV can also cause life-threatening pulmonary disease in bone marrow transplant recipients[4] and the elderly.[5–8] From 14 000 to 62 000 RSV-associated hospitalizations of the elderly occur annually in the United States.[8]

Although the importance of RSV as a respiratory pathogen has been recognized for >40 years, a vaccine is not yet available because of several problems inherent in RSV vaccine development. The peak of severe disease and mortality associated with pediatric RSV infection occurs in infants <3 months of age, who often have high titers of RSV maternally derived antibody. These young infants may not respond adequately to vaccination because of immunologic immaturity and/or suppression of the immune response by maternally derived antibody.[1,9–11] An RSV vaccine will also need to protect against the antigenically divergent groups A and B (see below). Most importantly the vaccine must not potentiate naturally occurring RSV disease, as was observed with the formalin-inactivated RSV vaccine (see below).[12–14] Because serious RSV disease can occur in high risk individuals who have experienced previous RSV infection as well as in RSV-naive infants, it is likely that more than one type of RSV vaccine will be needed to immunize all of those who would benefit from vaccination.

BACKGROUND

Epidemiology. By 2 years of age, almost all children will have been infected with RSV, and ~50% will have been infected twice.[15] Reinfection can occur throughout life and is usually symptomatic, although RSV infection does not generally cause lower respiratory tract disease in immunocompetent adults and healthy older children.[16]

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RSV epidemics occur yearly during winter and early spring in temperate climates and in the rainy season in some but not all tropical climates.[17, 18] RSV group A and group B viruses cocirculate during epidemics, although one may predominate.[19–22] Humans are the only known reservoir for RSV; spread of the highly contagious virus occurs by contaminated nasal secretions via large droplets rather than small particle aerosols, and close contact with an infected individual or contaminated environmental surface is required for transmission.[23] RSV can persist as a fomite on hard surfaces for several hours,[24, 25] and for this reason it is an important cause of nosocomial respiratory illness, particularly in pediatric wards.[25]

Immunity. Virus-specific immune responses are largely responsible for protection against RSV-associated LRI and recovery from RSV infection. Immunity to RSV is mediated via humoral and cellular effectors, including serum antibody (acquired as a result of infection or maternally derived in young infants), secretory antibody, and MHC class I-restricted cytotoxic T lymphocytes. The RSV F glycoprotein may also elicit innate immune responses via Toll-like receptors and CD14.[26, 27] Natural immunity to RSV is incomplete, and infection recurs throughout life, as has been demonstrated by epidemiologic[15, 28] and challenge studies in healthy young adults.[29] Healthy older children and adults, however, are usually protected against RSV-associated LRI. In general humoral immune responses (secretory and serum antibodies) appear to protect against infection of the upper and lower respiratory tract, respectively, whereas cell-mediated responses directed against internal proteins appear to terminate infection. Although the adoptive transfer of primed T cells will halt RSV replication in immunodeficient mice, the adoptive transfer of RSV-specific cytotoxic T lymphocytes may also potentiate disease.[30] These data, combined with more recent information regarding the role of the RSV G glycoprotein in disease pathogenesis,[31–34] suggest that there may be an immune component to RSV illness.

The role of local immunity in the protection of the upper respiratory tract against RSV is suggested by experimental data from studies in cotton rats[35–37] and adult volunteers[38] and by observational data from infants.[39] In adults the presence of secretory neutralizing antibody, but not serum antibody, correlated with protection of the upper respiratory tract against RSV infection.[38] In infants the development of IgA in nasal secretions correlated temporally with viral clearance after natural infection.[39]

RSV replicates exclusively in the respiratory epithelium. For this reason serum neutralizing antibody does not prevent infection, as it does for pathogens that produce viremia, such as measles and varicella. High titers of RSV serum neutralizing antibody protect the lower respiratory tract against RSV infection, however, as has been demonstrated by animal studies,[35–37] epidemiologic observations in infants and young children[40–43] and clinical trials of RSV hyperimmune globulin and monoclonal antibody in high risk infants.[44, 45]

Primary infection with RSV does not always elicit an immune response that will protect the lower respiratory tract, because RSV-associated LRI can occur in young children experiencing their second episode of RSV.[15, 43] Young infants develop levels of neutralizing antibody and F and G glycoprotein antibodies to RSV that are only 15 to 25% of those observed in older children.[46] The suboptimal response of young infants to primary infection with RSV has important implications for vaccine development, because it suggests that more than one dose of vaccine will likely be needed to induce adequate levels of RSV serum neutralizing antibody in this population.

VACCINE DEVELOPMENT

General considerations. A successful RSV vaccine should prevent serious RSV-associated lower respiratory tract illness in those at risk. The primary target populations for RSV vaccines are very young infants and the elderly, though older high risk children would also benefit from RSV immunization. It is likely that different vaccines will be needed for the various target populations; nonreplicating vaccines may be useful in the elderly, in high risk older children and for maternal immunization, but live virus vaccines are likely to be required for RSV-naive infants.

Past experience: formalin-inactivated RSV vaccine. The enhanced disease observed in infants who received inactivated RSV vaccine in the 1960s has profoundly impacted the current development of an RSV vaccine. This formalin-inactivated RSV vaccine (FI-RSV) was developed and administered to infants and children in the United States. During the winter of 1966 to 1967, immunized children were exposed to RSV in the community and those children who were seronegative for RSV before vaccination experienced a significant increase in the frequency and severity of RSV lower respiratory tract disease (bronchoconstriction and pneumonia) and greater incidence of hospitalization compared to control children. Tragically two immunized infants died as toddlers as a consequence of subsequent RSV infection.[12–14, 47] Autopsy material showed bronchopneumonia with atelectasis and pneumothoraces. Pulmonary histopathology was reported in the literature as a "peribronchiolar monocytic infiltration with some excess in eosinophils."[12]

Questions regarding the mechanism of illness associated with vaccine-enhanced RSV disease have dominated RSV literature for several decades. Interestingly recent evidence from rodent models suggests that bron-

choconstriction and pneumonia, the two main components of enhanced RSV disease, are mediated by different arms of the immune system. Immune complexes that fix complement in the lungs are critical for bronchoconstriction (SJ Hoffman, RA Karron, FR Laham, et al. Complement factor C5 regulates bronchoconstriction during enhanced respiratory syncytial virus disease by modulating expression of the C3a anaphylatoxin receptor, submitted for publication),[48] and bronchoconstriction is specifically mediated by the complement C3a anaphylotoxin and modulated by complement component C5 (SJ Hoffman, RA Karron, FR Laham, et al. Complement factor C5 regulates bronchoconstriction during enhanced respiratory syncytial virus disease by modulating expression of the C3a anaphylatoxin receptor, submitted for publication). Complement appears to play no role in enhanced RSV pneumonia, however. Rather this appears to be T helper cell (Th) 2 cell-mediated, because depletion of $CD4^+$ T cells and interleukin (IL)-4 or IL-10 decrease the cellular infiltrate in the lungs of BALB/c mice with enhanced RSV pneumonia.[49–52] The absence of a Th1-promoting RSV-specific cytotoxic T cell response after immunization with FI-RSV has been associated with the Th2 polarization observed during enhanced disease.[53, 54]

Although immunization of children with FI-RSV elicited anti-RSV antibodies, these antibodies were nonneutralizing and nonprotective.[12–14, 47] The high levels of nonneutralizing antibodies generated by FI-RSV suggested that formalin disrupted critical protective epitopes during the process of inactivation. However, other nonreplicating vaccines also elicited high levels of nonneutralizing antibodies and primed for pneumonia in rodents after RSV challenge,[32, 54] indicating that formalin inactivation may not be a critical step for the generation of a pathologic antibody response. Additional studies to elucidate the mechanisms responsible for the differences between antibodies elicited by replicating and nonreplicating RSV vaccines are in progress.

The clinical experience with FI-RSV and the information gleaned from animal models of disease enhancement suggest key features of an RSV vaccine for seronegative infants. The vaccine should induce protective levels of neutralizing antibody, as well as $CD8^+$ RSV-specific cytotoxic T cells, and a pattern of CD4 response like that evoked by wild-type (wt) RSV. Although a live attenuated vaccine is most likely to exhibit these characteristics,[9, 55] it is possible that novel immunization strategies that combine nonreplicating vaccines with cytokines or new adjuvants might achieve these goals.[56, 57]

Live attenuated RSV vaccines. Live attenuated vaccines may offer several advantages over nonreplicating vaccines, especially for RSV-naive infants and young children. Intranasal immunization with a live attenuated vaccine should induce both systemic and local immunity and therefore protect against upper as well as lower respiratory disease. Also the immune response to a live vaccine should closely resemble the response to natural infection and therefore not produce enhanced disease on exposure to wild-type virus.[9] Like other live attenuated intranasal respiratory virus vaccine candidates,[10, 11] a live intranasal RSV vaccine candidate has been shown to replicate in young infants in the presence of maternally acquired antibody.[58] This feature will be critical for the success of a live attenuated RSV vaccine in young infants. A live attenuated RSV vaccine would probably need to be administered in multiple doses to young infants.

As is the case with most live attenuated vaccines, achieving an appropriate balance between attenuation and immunogenicity is not straightforward, given that the levels of RSV vaccine virus replication tend to correlate with induction of immune responses and clinical symptoms.[1, 59] The level of reactogenicity that can be tolerated will depend on the age and condition of the target population. For example vaccine-induced nasal congestion would likely be acceptable in toddlers and the elderly but is unacceptable for very young infants, who are obligate nose breathers.[58] Although novel strategies using recombinant technology may ultimately be needed to produce an attenuated, immunogenic live RSV vaccine for young infants, recent studies of a biologically derived live attenuated parainfluenza type 3 vaccine demonstrate that a live respiratory virus vaccine that is sufficiently attenuated yet immunogenic in young infants can indeed be developed.[60]

Biologically derived live attenuated vaccines. Several strategies for the development of a live attenuated vaccine were originally explored, including the creation of host range mutants, cold-passaged (*cp*) mutants and temperature-sensitive (*ts*) mutants (which are unable to grow at high temperatures). Based on previous experience with live attenuated influenza vaccines,[61, 62] growth of the *cp* and *ts* mutants *in vivo* was expected to be restricted, particularly in the lower respiratory tract (at core body temperature). The clinical evaluation of mutants developed between 1968 and 1976 that were either *cp* or *ts* [designated *cp*RSV (Lot 3131), RSV *ts*-1 and *ts*-2] has been extensively summarized elsewhere.[9, 55, 63] In brief these vaccine candidates were either underattenuated (*cp*RSV and RSV *ts*-1) or overattenuated (RSV *ts*-2), and reversion to wild-type (ts^+) phenotype was observed in viral isolates obtained from infants and children who received the RSV *ts*-1 mutant. Transmission of the *ts*-1 mutant from vaccinated children to placebo recipients also occurred.[64, 65] Importantly enhanced disease was not observed when infants who

received RSV *ts*-1 or *cp*RSV were naturally infected with wild-type RSV.[64, 66] Although these early attempts to develop a live attenuated RSV vaccine were unsuccessful, they established the use of placebo-controlled, double blind trials with postvaccination surveillance through RSV epidemics as the model for future evaluation of live attenuated RSV vaccines in children. In addition *cp*RSV is the progenitor of the *cpts* RSV vaccines recently evaluated in children (Table 1 and this section).

Investigators in the United Kingdom developed three *ts* mutants of RSV A, which were derived from the RSS-2 strain by chemical mutagenesis of virus grown in the MRC-5 human diploid cell line (Table 1).[67, 68] Although attenuated in comparison with wt RSV, two of these mutants caused upper respiratory tract illnesses in adults and were therefore not sufficiently attenuated for further evaluation in children.[67] Replication of the third mutant was not associated with illness in adults,[68] but this candidate has not been evaluated in children.

A series of live attenuated RSV A *cpts* candidate vaccines were derived from further attenuation of *cp*RSV through chemical mutagenesis (Fig. 1). This process generated *ts* candidate vaccines with a range of shutoff temperatures (35–37°C) that displayed a spectrum of attenuation in rodents and nonhuman primates.[69–72] Each of these candidate vaccines was shown to protect chimpanzees against challenge with wt RSV,[59, 69, 70] and those recovered from chimpanzees and nude mice showed greater stability of the *ts* phenotype than had previously been observed with the *ts*-1 virus.[55]

Several of these candidate intranasally administered vaccines were evaluated in Phase I clinical trials (Table 1).[58, 59] *cpts* 248/955 and 530/1009 vaccines were evaluated sequentially in adults, RSV-seropositive children, and RSV-seronegative children as young as 6 months. Although both *cpts* 248/955 and 530/1009 were attenuated in adults and seropositive children, neither was sufficiently attenuated in seronegative children to permit evaluation in very young infants.[59] However,

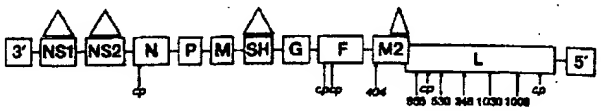

FIG. 1. Schematic representation (not to scale) of the mutations found in live attenuated RSV candidate vaccine viruses. Mutations in the RSV genome are identified as point mutations (*arrows*) or as gene deletions (Δ). Point mutations are further identified as mutations induced by serial cold-passage (*cp*), or as temperature-sensitive (*ts*) mutations induced by chemical mutagenesis (identified numerically). The number assigned to the *ts* mutation indicates the clone number of the virus in which the mutation was first identified.

the *cpts* 248/404 vaccine was subsequently evaluated in children and infants as young as 1 month old. *cpts* 248/404 was highly attenuated in these infants but caused nasal congestion that, in some instances, interfered with feeding and sleeping.[58] *cpts* 248/404 also induced serum neutralizing antibody and RSV IgG and IgA responses in children >6 months of age, whereas predominantly IgA responses were observed in the younger infants.[58] Surveillance conducted in the RSV season after vaccination did not demonstrate disease enhancement but provided preliminary evidence of protection against symptomatic RSV infection[58]: 98% (173 of 176) of the isolates maintained the full *ts* phenotype; 3 isolates from a single subject showed an alteration in phenotype accompanied by a nucleotide substitution at the 404 site.

Although the *cpts* 248/404 vaccine was not sufficiently attenuated for young infants, evaluation of this vaccine candidate provided important information regarding replication and immunogenicity of a live attenuated RSV vaccine in the presence of maternal antibodies, phenotypic stability of a *cpts* vaccine and preliminary evidence of protection against illness after wt RSV infection.[58]

Genetically engineered [complementary DNA (cDNA)-derived] live attenuated vaccines. The ability to recover infectious virus from cDNA clones of RSV[73] has provided insight into the genetic basis of attenuation of biologically derived vaccines and has-

TABLE 1. RSV vaccines evaluated in clinical trials since 1990

Vaccine	Composition	Manufacturer	Subjects	Status
RSV subunit				
BBG2Na	RSV G peptide-BB*	Pierre Fabre	Adults†	Inactive
PFP-1, 2, 3	Purified F protein	Wyeth	Adults, children‡	Inactive
RSV A subunit	Copurified F, G, M proteins	Aventis Pasteur	Adults	Active
RSV live attenuated				
ts-1A, B, C	RSV A *ts* mutants	MRC	Adults	Inactive
cp or *cpts*§	Serially passaged derivatives of *cp*RSV A2	NIH or Wyeth/NIH	Adults,† children, infants	Inactive
recombinant RSV	Recombinant derivatives of *cp*RSV A2¶	Wyeth/NIH	Adults, children, infants	Active

‡ Includes healthy adults, elderly, pregnant women and healthy and high risk RSV-seropositive children.

† Healthy adults and the elderly.

* BB is the albumin-binding domain of streptococcal protein G and functions as a carrier protein.

§ Includes *cp*RSV, which was evaluated in adults, and *cpts* 248/955, *cpts* 530/1009, *cpts* 530/1030 and *cpts* 248/404, each of which was evaluated in RSV-naive children as young as 6 months. *cpts* 248/404 was evaluated in infants as young as 1 month of age.

¶ Includes mutants with multiple *ts* mutations and/or deletions of non-essential genes.

tened the development of additional live attenuated vaccine candidates through the use of recombinant technology.[1, 74] Mutations present in *cp* RSV and six of its *ts* derivatives were inserted into wt RSV singly and in combination, and the majority of attenuating mutations were found to occur in the polymerase gene, with a notable exception being the 404 mutation in the *M* gene start signal (Fig. 1).[1, 74–76]) With this information attenuating mutations from biologically derived vaccines have been combined to produce further attenuated vaccine candidates.[1, 55, 74, 77] Deletion of a nonessential gene (*SH, NS1, NS2,* or *M2-2*[78]) in combination with known attenuating *cp* and *ts* mutations might also produce a highly attenuated, genetically stable vaccine (Fig. 1).[79, 80] Recombinant RSV vaccines containing *cp* and *ts* mutations and deletions of the *SH* or *NS2* gene are currently being evaluated in clinical trials.[81] Foreign genes can also be inserted into a recombinant RSV genome,[82] so that a cDNA-derived bivalent RSV vaccine might be developed that contained the *G* genes from RSV A and B.[82–84] Alternatively immunomodulating genes (granulocyte-macrophage colony-stimulating factor, for example) might be introduced in an effort to enhance immunogenicity in young infants.[82, 85–87]

Recombinant technology also provides the opportunity for creation of chimeric viruses containing the RSV F and G surface glycoproteins, with one or more internal genes provided by related respiratory viruses. For example, a chimeric vaccine containing the bovine parainfluenza type 3 (PIV-3) backbone, human PIV-3 surface glycoproteins, and RSV F and G glycoproteins induced an immune response to both human PIV-3 and RSV in rhesus monkeys.[88] Chimeric viruses containing genes from human and bovine RSV have also been created.[1, 89] Although native bovine RSV does not protect chimpanzees against human RSV challenge,[90] recombinant human/bovine chimeric vaccines may prove more successful.[1, 89]

Subunit vaccines. RSV F and G, the viral glycoproteins that induce neutralizing and protective antibodies,[1] have been evaluated as potential candidate vaccines. Subunit vaccines are most likely to be useful for immunization of the elderly and high risk children and might also be used for maternal immunization. Vaccines that have recently been evaluated in clinical trials include purified F glycoprotein (PFP) (PFP-1, PFP-2, and PFP-3[91–98]); copurified F, G and matrix (M) proteins[99]; and BBG2Na, a peptide from the G glycoprotein conjugated to the albumin-binding domain of streptococcal protein G (Table 1).[100–103] A chimeric RSV FG fusion protein vaccine was evaluated in Phase I trials in adults but is not being pursued further (S Holmes, personal communication).

RSV F subunit vaccines have been evaluated in healthy adults, in children >12 months with and without chronic underlying pulmonary disease (chronic lung disease of prematurity or cystic fibrosis), in institutionalized and ambulatory elderly subjects, and in pregnant women.[92–98, 104] These vaccines, designated PFP-1, PFP-2 and PFP-3, contain the RSV F glycoprotein purified by immunoaffinity column (PFP-1) or ion exchange chromatography (PFP-2 and PFP-3). PFP-1 and PFP-2 were prepared with wt RSV, and PFP-3 was prepared with a *cpts* RSV strain.[105] PFP-1 and PFP-2 are adsorbed to aluminum hydroxide, and PFP-3 is adsorbed to aluminum phosphate. The PFP vaccines have been well-tolerated in these populations: acute reactions were minimal and enhanced disease was not observed.[92–98, 104] In addition 50 μg of vaccine was the most immunogenic of the doses tested, and 4-fold or greater rises in RSV-neutralizing antibody titers were observed in ~50 to 75% of the vaccinees, depending on the levels of preimmunization neutralizing antibodies. A metaanalysis of PFP-1 and PFP-2 studies in adults and children concluded that these vaccines appeared to reduce the incidence of RSV infections, although the heterogeneity of the populations studied raised doubts about the validity of this conclusion. The incidence of RSV LRI was not significantly reduced.[106]

Most recently a Phase I study of PFP-2 was conducted in pregnant women, and a Phase II study of PFP-3 was conducted in children with cystic fibrosis. Thirty-five women in the 30th to 34th week of uncomplicated pregnancies were randomized to receive either 50 μg of PFP-2 vaccine or saline placebo. PFP-2 was well-tolerated and immunogenic in these women. All 35 infants were born healthy, and there was no difference in neonatal and perinatal outcomes between vaccine and placebo recipients. During RSV season there was no increase in the frequency or morbidity associated with respiratory illness in infants of vaccine recipients. The geometric mean titers of RSV F antibody were 4-fold higher in the children born of immunized mothers than those born of placebo recipients at birth, 2 months and 6 months of age. Fourfold increases in RSV neutralizing antibody titers were reported in 10% of immunized mothers and in their infants at birth and at 2 months of age.[98]

The study of PFP-3 vaccine in children with cystic fibrosis was conducted based on previous trials that demonstrated reduction in numbers of LRI episodes (although not in episodes of RSV infection) in 34 children with cystic fibrosis who received PFP-2 vaccine or placebo.[107] A group of 294 children 1 to 12 years of age with cystic fibrosis were immunized with 30 μg of PFP-3/aluminum phosphate vaccine or aluminum phosphate alone. The vaccine was safe, well-tolerated and immunogenic, with 67% of subjects showing a 4-fold rise in neutralizing antibody titer to RSV-A and 55% to RSV-B.[105] However, there were no statistically significant differences in the frequency of LRI episodes

in vaccine or placebo recipients (V Laposta, personal communication).

BBG2Na is a prokaryotically expressed fusion protein which consists of the central conserved region of the G glycoprotein from the RSV A Long strain (residues 130 to 230) fused to the albumin-binding domain of streptococcal protein G, which acts as a carrier protein.[101, 102, 108, 109] Residues 158 through 190 are conserved among RSV A isolates, and Residues 163 to 174 are conserved among RSV A and B isolates.[110] Despite induction of only modest levels of RSV-neutralizing antibody,[102] BBG2Na protected rodents against challenge with RSV, and sera from RSV-seropositive individuals was found to react with peptides derived from this region.[111] In Phase I trials 10-, 100- or 300-μg doses of BBG2Na in alum were well-tolerated in healthy young adults.[103] Four weeks after immunization, the 100- and 300-μg doses of vaccine induced $\geq$2-fold increases in neutralizing antibody in 33 to 71% of vaccinees.[103] In Phase II studies of this vaccine, two healthy young adults developed type III hypersensitivity (purpura). An efficacy trial of BBG2Na in the elderly was recently completed.

A subunit vaccine consisting of copurified F, G and M proteins from RSV A has been administered intramuscularly to healthy adults with either alum or polydicarboxylatophenoxyphosphazene, an adjuvant (Table 1).[99] Both formulations of the vaccine were well-tolerated and comparably immunogenic, with 2-fold and 4-fold increases in neutralizing antibody titers detected in 96 to 100% and 76 to 83% of vaccinees, respectively. In this previously primed population, neutralizing antibody responses to RSV A and RSV B were detected with comparable frequency. Studies of this vaccine in other populations are in progress.

CONCLUSIONS

In the past decade tremendous progress has been made in the development of RSV vaccines. Currently two types of candidate vaccines are being evaluated in clinical trials: subunit vaccines for immunization of the elderly, RSV-seropositive children at high risk for severe RSV disease and pregnant women; and live attenuated vaccines which would be used primarily for immunization of young infants and perhaps the elderly. It is possible that combinations of different types of vaccines might be needed for certain populations. For example optimal immune responses in the elderly might result from simultaneous administration of a live and a nonreplicating RSV vaccine.[104] The use of recombinant technology should allow further refinement of existing live attenuated candidate vaccines to produce engineered vaccines that are satisfactorily attenuated, immunogenic and phenotypically stable. This will undoubtedly be an iterative, empiric process, with data from clinical trials used to determine the relative value of available attenuating mutations.

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DISCUSSION

Question: The obstacles towards trying to vaccinate the tiny group of children who are at highest risk for RSV seem almost insurmountable, especially when you talk about the very tight balance between obtaining enough replication to be immunogenic, and too much replication. Is anyone thinking about strategies involving immunization of contacts rather than the child, given that most of the infections in that very young age group are probably transmitted within the family?

Ruth A. Karron, M.D.: It is a potential strategy. Here are the issues: Most live virus vaccines that are attenuated enough for young infants don't infect older children, so you can't immunize those sibling contacts. And conversely, if you were going to develop a live attenuated vaccine that was sufficiently immunogenic and infectious to be effective in those sibling contacts, I think there is a chance that the attenuated virus could be transmitted to the young infants; that would be worrisome.

The other potential alternative is to develop a subunit vaccine. However, the data we've seen so far suggest that the immune response to those is certainly going to be short lived; you would have to immunize contacts every year.

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Live-attenuated intranasal parainfluenza virus type 2 vaccine candidates developed by reverse genetics containing L polymerase protein mutations imported from heterologous paramyxoviruses

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Abstract

Live-attenuated recombinant human parainfluenza virus type 2 (rHPIV2) vaccine candidates were created using reverse genetics by importing known attenuating mutations in the L polymerase protein from heterologous paramyxoviruses into the homologous sites of the HPIV2 L protein. Four recombinants (rF460L, rY948H, rL1566I, and rS1724I) were recovered and three were attenuated for replication in hamsters. The genetic stability of the imported mutations at three of the four sites was enhanced by use of alternative codons or by deletion of a pair of amino acids. rHPIV2s bearing these modified mutations exhibited enhanced attenuation. The genetically stabilized mutations conferring a high level of attenuation will be useful in generating a live-attenuated virus vaccine for HPIV2.
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Keywords: Parainfluenza virus; Paramyxovirus; L polymerase

1. Introduction

Human parainfluenza virus type 2 (HPIV2) is an important respiratory pathogen causing croup and bronchiolitis in infants and young children worldwide. HPIV1, HPIV2, and HPIV3 account for, approximately, 6, 3, and 11%, respectively, of pediatric hospitalizations due to respiratory disease [1]. Together, these three HPIV serotypes nearly equal respiratory syncytial virus (RSV) in the level of disease burden [1]. Vaccines for the three HPIVs and RSV are not available, but efforts are underway using reverse genetics to develop recombinant live-attenuated RSV and PIV vaccines for intranasal immunization of infants and young children [2–5].

HPIV2 is a single stranded negative-sense RNA virus and is a member of the *Paramyxoviridae* family. HPIV2 is classified in the *Rubulavirus* genus, whereas HPIV1 and HPIV3 are members of the *Respirovirus* genus. The genome of the V94 clinical isolate of HPIV2 (HPIV2/V94), the parent of the recombinant viruses described in this report, is 15,654 nucleotides in length and conforms to the "rule of six", which is the requirement that the virus genome length be evenly divisible by six [6–9]. The genetic organization of HPIV2 is similar to that of SV5 and mumps virus, which are prototypic members of the *Rubulavirus* genus, except that HPIV2 lacks an SH gene [9]. The HPIV2 genome encodes seven polypeptides from six genes. The ribonucleocapsid-associated polypeptides include the nucleocapsid protein (N), the phosphoprotein (P), and the large polymerase (L) protein that carry out transcription and replication. Similar to other Rubulaviruses, the P/V gene of HPIV2 contains an alternative open reading frame (ORF) that is accessed by co-transcriptional editing. The unedited mRNA encodes the accessory V protein, whose functions include inhibition of the host interferon response [10,11], whereas the edited mRNA encodes the P protein [9]. The internal matrix protein (M),

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the fusion protein (F), and the hemagglutinin–neuraminidase attachment protein (HN), are the envelope-associated proteins. The HN and F proteins of the HPIVs induce neutralizing antibodies and are the major protective antigens [12]. The gene order for HPIV2 is 3′-N-P/V-M-F-HN-L-5′ [12].

Previous analysis of several paramyxovirus mutants conferring temperature sensitive (*ts*) and attenuation (*att*) phenotypes identified the large polymerase (L) protein as a major viral target for mutagenesis, useful in the development of vaccines [2,13,14]. The wild type amino acid assignments at the positions of some of the L protein mutations, which specified an *att* phenotype identified to date in various RSV, HPIV1, or HPIV3 mutant viruses are conserved among many paramyxoviruses, suggesting that importation of a mutation specifying an *att* phenotype involving a conserved assignment into the homologous site of a heterologous paramyxovirus might confer an *att* phenotype [3,5,14,15]. This has proven to be the case, and it has been found that mutations specifying an *att* phenotype can be imported from an attenuated paramyxovirus into heterologous paramyxoviruses to rapidly yield a vaccine candidate with *ts* and *att* phenotypes [14,16,17]. Furthermore, importation also can be successful at sites where the wild type amino acid assignment is not highly conserved [14,18], as will be described in this report.

It is important that the *att* phenotype of a live-attenuated vaccine virus is stable. Typically, missense mutations identified in nature differ from the wild type by a single nucleotide substitution, and, thus, can readily revert, given the high mutation rate of RNA viruses. We recently described a strategy to increase the stability of missense mutations specifying *att* phenotypes, which was demonstrated with two mutations in the L protein of recombinant HPIV1 [18]. By that strategy, codons were identified that would require three nucleotide substitutions to change the codon to one that specified the wild type phenotype. These codon substitution mutations were referred to as "stabilized" mutations. Stabilization was confirmed by monitoring the stability of the *ts* phenotype during serial passage in vitro at increasing temperature [18]. In several instances, these stabilized codons also manifested an increased level of temperature sensitivity and attenuation compared to the mutants bearing the original imported mutations and, thus, provided a method for optimizing the attenuation phenotype [18]. In the present study, we applied these techniques of importation of mutations specifying an *att* phenotype and their subsequent codon stabilization and optimization to the generation of HPIV2 vaccine candidates. A second strategy to enhance the genetic stability of the imported mutations was also employed, in which a two-amino acid deletion was introduced at the site of the imported mutation. Utilizing these two approaches, we were able to identify stabilized codon substitution and deletion mutations that attenuated HPIV2 for hamsters. To the best of our knowledge, attenuating mutations and attenuated derivatives of HPIV2 have not been previously reported.

2. Materials and methods

2.1. Cells lines and viruses

HEp-2 (ATCC CCL 23) and LLC-MK2 (ATCC CCL 7.1) cells were maintained in OptiMEM I (Life Technologies, Gaithersburg, MD) supplemented with 5% FBS and gentamicin sulfate (50 ug/mL). HPIV2 strain V9412-6 (HPIV2/V94) was isolated from a nasal wash specimen provided by Dr. Peter Wright of Vanderbilt University. HPIV2/V94 was biologically cloned and amplified on Vero cells for a total of nine passages [19], followed by amplification with two to four passages on LLC-MK2 cells. The HPIV2/V94 virus that is derived from cDNA contains an introduced *Not*I restriction enzyme site in the upstream non-translated region of the N gene (HPIV2 nucleotide positions 149–156) and is designated rV94Not. This recombinant and the biologically-derived parent virus serve as control viruses in these studies. The recombinant HPIV2 (rHPIV2) was grown in LLC-MK2 cells, as described previously [9,19]. Viruses were quantified by titration using serial 10-fold dilutions on LLC-MK2 cells, and virus-infected wells were identified by hemadsorption with guinea pig erythrocytes, as previously described [4,20].

2.2. Sequence comparisons to identify the homologous regions in HPIV2 for importation of mutations from RSV, HPIV3, or BPIV3

The predicted L polymerase sequences of HPIV2 and other paramyxoviruses were aligned using Clustal W alignment [21] with the MacVector program (Accelrys, San Diego, CA) and the GAP program of the Wisconsin Package Version 10.2 (Accelrys). The protein sequence of the L protein of HPIV2/V94 (GenBank accession no. AF533010) was compared to that of HPIV3 (GenBank accession no. Z11575), HPIV1 (GenBank accession no. AF457102), RSV (GenBank accession no. P28887), or to the BPIV3 Kansas strain (GenBank accession number AF178654) to identify the homologous positions of each of the attenuating mutations identified in the heterologous paramyxoviruses. The specific changes made at each position in the nucleotide and amino acid sequence of L in rHPIV2 are indicated in Fig. 1 and Table 1.

2.3. Construction of mutations in rHPIV2 cDNA

Mutations were introduced into the HPIV2/V94 antigenomic sense cDNA. This cDNA (designated pFLC V94Not) contains a *Not*I restriction site that was introduced between nucleotides 149 and 156 (GCGGCCGC) by PCR mutagenesis using standard molecular cloning techniques. The *Not*I restriction site was introduced to aid in subsequent cloning steps and is present in each of the recombinant HPIV2 mutants generated. The L gene mutations were introduced by a two-step PCR mutagenesis protocol [22] using the Advantage-HF PCR kit (Clonetech Laboratories, Palo Alto,

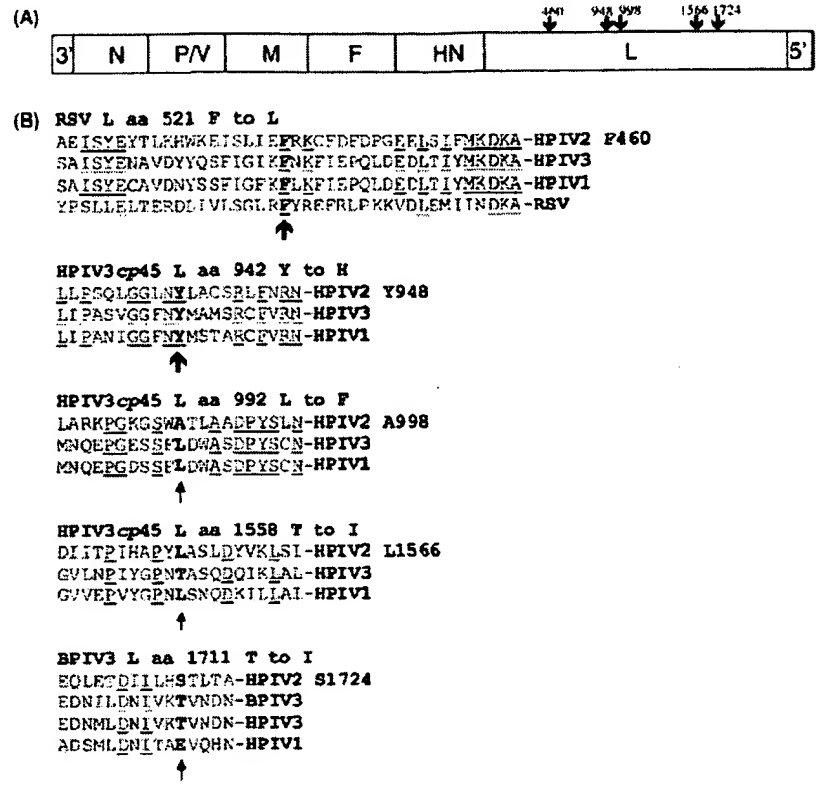

Fig. 1. (A) Schematic representation of the HPIV2 genome indicating the relative positions of the five mutagenized sites in the L protein at amino acid sequence positions 460, 948, 998, 1566, and 1724. (B) Sequence alignments of regions of the L proteins of the HPIV2, HPIV3, BPIV3, HPIV1 or RSV wild type viruses at the sites of the five mutations. The position of the HPIV2 amino acid selected for mutation is in bold and areas of identity between HPIV2 and the heterologous viruses are underlined. Large upward pointing arrows indicate mutation sites where the wild type amino acid assignment is highly conserved among the paramyxoviruses shown whereas small upward pointing arrows indicate sites where the amino acid assignment is less well conserved. The sequence position and amino acid substitution in the original attenuated heterologous paramyxovirus parent is given above each sequence alignment and the position of the imported mutation in the HPIV2 L is given to the right of the HPIV2 sequence.

CA). The region of the L open reading frame (ORF) that had been amplified during PCR mutagenesis was sequenced in its entirety following introduction into pFLC V94Not using a Perkin-Elmer ABI 3100 sequencer with the BigDye sequencing kit (Perkin-Elmer Applied Biosystems, Warrington, UK) to confirm that the amplified region had not acquired adventitious mutations during PCR amplification. Each deletion mutation that was introduced consisted of a six-nucleotide deletion encompassing two contiguous amino acids, thereby, preserving the polyhexameric length of the genome (Table 1).

2.4. Recovery of rHPIV2 mutant viruses

Recovery of each mutant rHPIV2 was performed at the permissive temperature of 32 °C, as described previously for rHPIV2 [9]. The rHPIV2 mutants were biologically cloned either by two rounds of plaque purification or by serial terminal dilution on LLC-MK2 monolayer cultures. To confirm that recovered recombinant viruses contained the appropriate mutations, viral RNA (vRNA) was isolated and was RT-PCR amplified using the SuperScript First-Strand Synthesis System (Invitrogen Inc.) for the RT step and the Advantage cDNA PCR Kit (Clontech Laboratories) for the PCR step, according to protocols specified by the manufacturer, as previously described [9,23]. Reactions lacking reverse transcriptase were included to confirm that the template for the amplified product was RNA [18]. Sequence analysis was performed as described above.

2.5. Replication of HPIV2 in vitro (multicycle growth curves)

Wild type and mutant rHPIV2s were individually inoculated onto LLC-MK2 cell monolayers in six-well plates in triplicate at a multiplicity of infection (m.o.i.) of 0.01, and cultures were incubated at 32 °C for 8 days. 0.5 ml of medium from each well was harvested and replaced with 0.5 ml of fresh medium at 0 h and at 24 h intervals for 8 days post-infection. Virus present in the samples was quantified by titration on LLC-MK2 monolayers in 96-well plates incubated for 6 days at 32 °C. Virus was detected by hemadsorption with guinea pig erythrocytes [9,24], and the titer is

Table 1
Missense and deletion mutations introduced into the L ORF of HPIV2

Virus	Amino acid		Codon		Number of nucleotide changes to revert to wild type[b]	Viable virus recovered
	Wild Type	Mutant	Wild type	Mutant[a]		
rF460L[c]	Phe	Leu	TTT	CTG	2	+
rF460A[d]	Phe	Ala	TTT	GCA	3	+
rF460P[d]	Phe	Pro	TTT	CCA	3	+
rF460R[d]	Phe	Arg	TTT	CGA	3	−
r460del	Glu–Phe	Deletion	GGA-TTT	459/460	6[e]	−
rY948H[c]	Tyr	His	TAC	CAC	1	+
rY948A[d]	Tyr	Ala	TAC	GCA	3	+
rY948L[d]	Tyr	Leu	TAC	CTA	3	+
rY948G[d]	Tyr	Gly	TAC	GGA	3	+
r948del	Tyr–Leu	Deletion	TAC-CTC	948/949	6[e]	−
rA998F[c]	Ala	Phe	GCC	TTC	2	−
rA998C[d]	Ala	Cys	GCC	TGT	2	−
rA998N[d]	Ala	Asn	GCC	AAT	2	−
r998del	Ala–Thr	Deletion	GCC-ACC	998/999	6[e]	−
rL1566I[c]	Leu	Ile	TTG	ATC	1	+
rL1566A[d]	Leu	Ala	TTG	GCT	2	−
rL1566G[d]	Leu	Gly	TTG	GGT	2	−
rL1566K[d]	Leu	Lys	TTG	AAA	2	−
rL1566N[d]	Leu	Asn	TTG	AAT	2	−
r1566del	Leu–Ala	Deletion	TTG-GCT	1565/1566	6[e]	−
rS1724I[c]	Ser	Ile	TCT	ATT	1	+
rdel1724	Ser–Thr	Deletion	TCT-ACT	1724/1725	6[e]	+

[a] Missense mutations are indicated by the mutant codon; nucleotides that differ from any codon specifying the wild type assignment are underlined. Deletion mutations are indicated by the amino acid sequence positions of the deleted residues.
[b] Number of nucleotide changes required to revert to a codon specifying the indicated wild type amino acid.
[c] Recombinants bearing the original imported mutation corresponding to L protein mutations in RSV, HPIV3, and BPIV3 (see Fig. 1).
[d] Recombinants bearing novel codon substitution assignments.
[e] The insertion of six nucleotides would be required to restore the two deleted codons.

reported as $\log_{10}$ $TCID_{50}$/ml (50% tissue culture infectious dose/ml).

2.6. Replication of mutant rHPIV2s in LLC-MK2 cells at permissive and restrictive temperatures

The level of temperature sensitivity of replication of each rHPIV2 mutant was determined by comparing its level of replication to that of rHPIV2 at the permissive temperature of 32 °C and at the higher temperatures of 37–40 °C, as previously described [25]. Briefly, each virus was serially diluted 10-fold in 96-well LLC-MK2 monolayer cultures in L-15 medium (Gibco–Invitrogen Inc. Grand Island, NY) containing antibiotics. Replicate plates were incubated at the temperatures indicated above for 6–7 days, and virus-infected cultures were detected by hemadsorption with guinea pig erythrocytes. Virus titer at each temperature was determined in two to three separate experiments and is expressed as the $\log_{10}$ $TCID_{50}$/ml. The reduction in titer at elevated temperature compared to the titer at 32 °C was determined, and a mean reduction in titer was determined. The shut-off temperature is defined as the lowest temperature at which the reduction in virus titer compared to its titer at 32 °C was 100-fold greater than that of wild type rHPIV2 (designated rV94Not) at the same temperature.

2.7. Replication of rHPIV2 mutants in the respiratory tract of hamsters

Four- to five-week-old Golden Syrian hamsters (Charles River Laboratories, NY) were inoculated intranasally with 0.1 ml of L-15 medium containing $10^{6.0}$ $TCID_{50}$ of a wild type or mutant HPIV2. Lungs and nasal turbinates were harvested on day 4 post-infection, and the virus titer of tissue homogenates was determined by limiting dilution, as previously described [9,23]. The mean $\log_{10}$ $TCID_{50}$ per gram of tissue homogenate was calculated for each group of six hamsters. For the rV94Not group, the mean was calculated from three separate experiments to give a total of 18 hamsters.

3. Results

3.1. Importation of ts attenuating mutations identified in the L proteins of heterologous paramyxovirus mutants into homologous sites in rHPIV2

To generate attenuated HPIV2 derivatives, five *ts* attenuating missense mutations that were previously identified in the L polymerase proteins of three heterologous paramyxoviruses (RSV, HPIV3, or BPIV3) were imported into the

homologous sites in the HPIV2 L protein (Fig. 1, Table 1). The homologous sites were identified by alignment of the amino acid sequence of the L protein of HPIV2 with that of HPIV1, HPIV3, BPIV3 or RSV (Fig. 1B). One of the mutations that was imported into the HPIV2 L protein, F460L, was originally identified as a *ts* attenuating mutation (F521L) in the RSV*cpts*530 vaccine candidate [26–28] (Fig. 1B). Importation of this mutation into the homologous positions of HPIV3 (F456L) and HPIV1 (F456L) was previously found to attenuate these viruses [5,14]. Three other mutations that were imported into the HPIV2 L protein, Y948H, L998F, and L1566I, were originally identified as *ts* attenuating mutations in the L protein of the vaccine candidate HPIV3*cp*45 (Y942H, L992F, and L1558I) [15,16] (Fig. 1B). The fifth mutation that was imported into the HPIV2 L protein, S1724I, was originally identified as a *ts* attenuating mutation in the BPIV3 L protein (S1711I) (Fig. 1B) [3]. Of the five positions, only 460 and 948 had wild type amino assignments that were highly conserved among multiple paramyxoviruses, whereas the wild type amino acid assignments at positions 998, 1566 and 1724 were not highly conserved; indeed, those at 1566 and 1724 were not even conserved between HPIV1 and HPIV3, members of the same *Respirovirus* genus (Fig. 1B).

Viable mutant rHPIV2 viruses were recovered for four of the five imported mutations, namely F460L, Y948H, L1566I, and T1724I. A recombinant HPIV2 with the L998F mutation, corresponding to the HPIV3*cp*45 H992F mutation, could not be recovered (Table 1). The rHPIV2 mutants are designated by the specific mutation introduced, e.g., rF460L is recombinant HPIV2 mutant containing the imported F460L mutation.

3.2. Introduction of codon substitution mutations in HPIV2 L

We also sought to generate mutant rHPIV2s containing alternative amino acid assignments at some of these mutagenized positions. In the case of positions 460 or 948, alternative assignments were made using codons that would require three nucleotide substitutions in order to change to a codon specifying the wild type amino acid at the site. At position F460, two viable mutants requiring three nucleotide substitutions in order to revert back to the wild type assignment were obtained, namely r460A and r460P (Table 1). A recombinant bearing the F460R codon substitution mutant was not recovered, suggesting that this mutation may be lethal or highly restrictive. At position 948, three codon substitution mutants were recovered (rY948A, rY948L, rY948G) (Table 1).

In the case of position 998, for which the original imported mutation (A998F) could not be recovered into viable virus, two other amino acid assignments (A998C and A998N) not only were evaluated but also could not be recovered (Table 1). This suggests that, unlike HPIV1 and HPIV3, substitution mutations at this position in HPIV2 might not be viable. At position 1566, four other amino acid assignments were evaluated (L1566A, L1566G, L1566K, and L1566N), but only the rHPIV2 mutant bearing the original substitution (L1566I) was viable (Table 1).

3.3. Introduction of deletion mutations at sites of imported mutations

We also employed a second approach to generate attenuated HPIV2 derivatives with a very low probability for reversion, namely, deletion of the codon in question. In each case, an adjacent codon also was deleted in order to maintain the polyhexameric length of the genome. A deletion was engineered for each of the five mutant sites, but only the deletion involving codons 1724 and 1725 yielded a viable recombinant virus (Table 1).

Thus, six HPIV2 mutants with stabilized missense or deletion mutations were recovered, namely, r460A, r460P, r948A, r948L, r948G, and rdel1724. These were characterized for in vitro and in vivo growth properties in parallel with the four original importation mutants (rF460L, rY948H, rL1566I, and rS1724I).

3.4. Multicycle replication of rHPIV2 L gene mutants in vitro

The level of replication of each of the recovered rHPIV2 mutants in LLC-MK2 cells at 32 °C was compared to that of the parental recombinant rV94Not virus and the original biologically-derived clinical isolate HPIV2/V94. The mutant and parent viruses were all found to grow to high titer ($\geq 10^{6.8}$ $\log_{10}$ $TCID_{50}$/ml) (Table 2), indicating that the L

Table 2
Replication of wild type and mutant HPIV2s at permissive and restrictive temperatures in LLC-MK2 cells in vitro

Virus[a]	Mean titer at 32 °C	Mean $\log_{10}$ reduction in virus titer at the indicated temperature (°C)[b]			
		37	38	39	40
V94	7.2	0.0	0.3	0.4	0.5
rV94Not	7.5	−0.2	−0.1	0.3	0.5
rF460L	7.5	0.0	-0.3	0.6	1.1
rF460A	7.0	0.7	1.1	2.1	**5.0**
rF460P	6.8	1.3	1.1	2.2	**5.1**
rY948H	8.1	0.2	0.5	1.0	1.8
rY948A	7.2	0.1	0.6	**3.0**	**5.7**
rY948G	7.4	−0.1	0.5	0.9	3.1
rY948L	7.1	1.1	1.9	**5.5**	**5.7**
rL1566I	7.6	0.4	0.6	0.8	0.9
rS1724I	7.6	−0.3	0.5	**2.9**	**5.8**
rdel1724	7.3	1.0	1.5	**4.0**	**6.2**

[a] V94 is the biologically-derived wild type HPIV2 clinical isolate, and rV94Not is a recombinant version that contains a *Not*I site in the upstream noncoding region of the N gene. See Table 1 for a description of the other viruses. Viruses in bold have a *ts* phenotype.

[b] Values are the mean of at least two experiments. Values in bold type indicate temperatures at which the mean $\log_{10}$ reduction vs. 32 °C was ≥ 2.0 $\log_{10}$ compared to that of wild type rHPIV2. The lowest temperature in bold is the shut-off temperature.

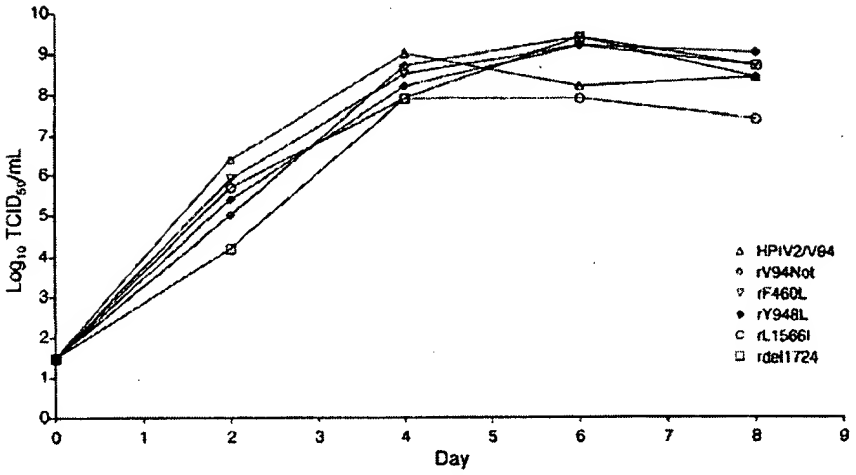

Fig. 2. The kinetics of multicycle replication of HPIV2 wild type and the indicated mutant viruses in LLC-MK2 monolayer cell cultures at 32 °C. LLC-MK2 cultures were inoculated in triplicate at an input m.o.i of 0.01 PFU per cell, and aliquots were taken at 24-h intervals, flash frozen, and analyzed later in parallel by limiting dilution to determine the level of replication. The mean level of replication for each time point is indicated.

protein mutations were not attenuating for replication at permissive temperature (32 °C) in vitro. A subset of these mutants (rF460L, rY948L, rL1566I, and rdel1724) was selected for evaluation of multicycle replication in LLC-MK2 cells at 32 °C. The kinetics and peak level of replication of these mutants were similar to those of rV94Not and HPIV2/V94 and each reached a peak titer (between 7.9 and 9.4 $\log_{10}$ $TCID_{50}$/ml) between days 4 and 6 post-infection (Fig. 2). This confirmed that these mutant recombinants are not attenuated for replication in cell culture at permissive temperature.

3.5. Evaluation of the temperature sensitivity of rHPIV2 L gene mutants in vitro

The ability of the mutant rHPIV2s to replicate on LLC-MK2 cell monolayers at elevated temperatures (37–40 °C) was examined (Table 2) to determine if they possess the *ts* phenotype. Interestingly, only one of the four original imported mutations (S1724I) conferred a *ts* phenotype even though all four of these mutations conferred the *ts* phenotype in the original viruses from which they were imported. In contrast, each of the six mutant recombinants bearing the alternative codon substitutions or deletion mutation (rF460A, rF460P, rY948A, rY948G, rY948L, and rdel1724) was *ts* and had a shut-off temperature of 39 or 40 °C (Table 2).

3.6. Replication of the HPIV2 L gene mutants in hamsters

The level of replication of the parent and mutant rHPIV2s in the upper and lower respiratory tract of hamsters was examined, since hamsters are a convenient small animal model that supports moderate levels of HPIV2 replication in the upper and lower respiratory tract following intranasal administration [9]. Hamsters in groups of six were inoculated intranasally with 0.1 ml of L-15 medium containing $10^{6.0}$ $TCID_{50}$ of parent or mutant HPIV2. On day 4 post-infection, the lungs and nasal turbinates were harvested, and the titer of virus in the tissue homogenates was determined. As shown in Table 3, recombinants representing three of the original imported mutations (rF460L, rL1566I, and rS1724I) were attenuated for replication in the upper or lower respiratory tract. The remaining original imported mutation, Y948H, did not attenuate HPIV2 for replication in hamsters even though this mutation specified an *att* phenotype in its original HPIV3 parent and in HPIV1 (following importation from HPIV3). Thus, importation of *att* mutations identified in heterologous viruses into HPIV2 conferred an *att* phenotype in three of the four viable recombinants.

The rHPIV2 mutants bearing the codon substitution mutations were more attenuated than the rHPIV2 mutants bearing the respective original imported mutation (Table 3). For example, the rF460A and rF460P mutants were highly attenuated in both the upper and lower respiratory tract whereas rF460L, which bears the original imported mutation, was only moderately attenuated and only in the lower respiratory tract. Similarly, rY948A, rY948G, and rY948L were attenuated in both the upper and lower respiratory tract whereas rY948H, which bears the original imported mutation, was not significantly attenuated at either site. Thus, the five viable modified codon substitution mutants exhibited an increased level of attenuation in comparison to rHPIV2s bearing the original imported mutation.

The rdel1724 mutant was highly attenuated in both the upper and lower respiratory tract of hamsters and was more attenuated at both sites than the original missense rS1724I

Table 3
Replication of wild type and mutant HPIV2s in the upper and lower respiratory tract of hamsters

Virus[a]	No. of animals	Mean virus titer[c] (log_{10} $TCID_{50}$/g ± S.E.)		Phenotype summary[d]	
		Nasal Turbinates	Lungs	*ts*	*att*
V94	6	4.9 ± 0.1	5.9 ± 0.4	–	–
rV94Not	18[b]	5.3 ± 0.1	5.3 ± 0.2	–	–
rF460L	6	5.0 ± 0.1	**3.1 ± 0.3**[e]	–	+
rF460A	6	**1.7 ± 0.2**	**≤1.5 ± 0.0**	+	+
rF460P	6	**1.6 ± 0.1**	**≤1.5 ± 0.0**	+	+
rY948H	6	5.6 ± 0.1	4.5 ± 0.4	–	–
rY948A	6	**3.9 ± 0.2**	**2.2 ± 0.2**	+	+
rY948G	6	**3.5 ± 0.3**	**2.2 ± 0.3**	+	+
rY948L	6	**1.7 ± 0.1**	**≤1.5 ± 0.0**	+	+
rL1566I	6	4.6 ± 0.4	**3.1 ± 0.5**	–	+
rS1724I	6	**3.5 ± 0.3**	**2.2 ± 0.2**	+	+
rdel1724	6	**2.4 ± 0.2**	**≤1.5 ± 0.0**	+	+

[a] Hamsters in groups of six were inoculated intranasally with 10^6 $TCID_{50}$ of the indicated virus. Nasal turbinates and lung tissues were harvested on day 4. Virus present in the tissues was quantified by serial dilution on LLC-MK2 monolayers at 32 °C.

[b] Total number of animals from three independent experiments.

[c] Data shown were compiled from three separate experiments, each of which had an rV94Not group as a control. The mean virus titer per gram of tissue for each group of animals receiving the same inoculum is shown. S.E., standard error.

[d] Summary of virus phenotype, either temperature sensitive (*ts*) or attenuating (*att*). (+) and (–) designate the presence or absence, respectively, of the specified phenotype.

[e] Bolded values are significantly decreased from both V94 and rV94Not with a p-value < 0.01. The significance and p-values were determined by using the Tukey–Kramer multiple comparisons test.

importation mutant. Since rdel1724 contains a two-amino acid deletion, it should not be able to readily revert following replication in vitro or in vivo, whereas rS1724I, which contains a single nucleotide substitution relative to wild type, has a substantially higher probability for reversion. Importantly, rdel1724 replicates efficiently at 32 °C (Fig. 2), indicating that the deletion mutation restricts replication in vivo without significantly altering replication in vitro at permissive temperature, an essential property of an attenuating mutation to be selected for incorporation into a vaccine candidate. These findings indicated that the level of attenuation for the upper and lower respiratory tract of hamsters that is conferred by a mutation imported into HPIV2 from a heterologous virus can be enhanced by use of codon substitution mutations or by deletion of a pair of amino acids at the site of an attenuating point mutation.

4. Discussion

The use of reverse genetics for engineering live-attenuated vaccine viruses permits the generation of live-attenuated intranasal vaccine candidates bearing defined attenuating mutations. In this report, reverse genetics was used to import attenuating mutations present in the L proteins of RSV, HPIV3, or BPIV3 to homologous sites in the L protein of HPIV2 yielding attenuated derivatives of HPIV2. In previous work, importation of attenuating mutations from heterologous viruses has been used to develop attenuating mutations for HPIV3 and HPIV1. For example, a mutation in the RSV L protein was introduced into the HPIV3*cp*45 vaccine candidate, rendering it more attenuated in vivo and more phenotypically stable following replication in vivo [5]. Importation of the mutation from RSV was, thus, used to fine-tune the level of attenuation of the HPIV3*cp*45 vaccine candidate. As a second example, a non-*ts* *att* mutation present in the C protein of Sendai virus [29,30], the murine counterpart of HPIV1, was imported into both HPIV3 and HPIV1 wild type viruses, yielding non-*ts* *att* derivatives [5,14,17]. In addition, *ts* mutations in L were imported from RSV and HPIV3*cp*45 to HPIV1, yielding attenuated *ts* derivatives [14]. The panel of mutations identified in this way that attenuate rHPIV1 are presently being used in combination to engineer live-attenuated HPIV1 candidates for clinical evaluation [39].

Importation of five independent attenuating missense mutations from RSV, HPIV3, or BPIV3 into HPIV2 identified: (i) sites that yielded viable rHPIV2 mutants; and (ii) sites at which the imported mutations rendered rHPIV2 attenuated. Four of the five sites of importation, at amino acid sequence positions 460, 948, 1566, and 1724, yielded viable viruses, indicating that these sites were amenable to mutation. Thus, amino acid alignment appeared to be effective in identifying sites for importation to yield viable virus. Three of the four viable imported mutants bearing imported mutations were attenuated in hamsters, specifically, those involving positions 460, 1566, and 1724. However, although each of these three attenuating mutations was both a *ts* and *att* mutation in the original heterologous paramyxovirus parent, only one, the

S1724I mutation, rendered HPIV2 *ts*. Thus, the strategy of importing attenuating mutations from heterologous viruses frequently is successful, but achieving the full phenotype of the original mutation is somewhat unpredictable. Perhaps, it is not surprising that differences in phenotype were observed between the heterologous parent viruses and the rHPIV2 derivatives, since the L protein of HPIV2 shares less than 33% amino acid sequence identity with RSV, HPIV3, or BPIV3. However, the major findings were that sites were identified in HPIV2 that yielded viable mutants and that a mutation in three of four of these sites yielded attenuated rHPIV2 mutants. This made it possible to next introduce codon substitution mutations at these sites to achieve further attenuation and increased genetic stability of the rHPIV2 mutant viruses.

Among the four sites for which viable recombinants were obtained, two involved wild type amino acid assignments that were highly conserved among heterologous paramyxoviruses (positions 460 and 948) and two involved poorly conserved wild type assignments (positions 1566 and 1724), for which the wild type assignment was not even conserved between HPIV1 and HPIV3, both members of the *Respirovirus* genus. Thus, importation need not involve a highly conserved assignment in order to yield viable virus. The original imported mutation was attenuating in three cases: F460L, L1566I, and S1724I; this also did not follow a pattern with regard to the conservation of the original wild type assignment. This suggests that the presence of a highly conserved wild type assignment at the position of importation was not a predictor of whether the import would be attenuating and, at least in these examples, does not appear to be an important consideration in choosing sites for importation. Thus, the identification of homologous sites by sequence alignment is guided by local sequence relatedness and need not involve conservation of the exact amino acid position.

Maintaining the stability of the attenuation phenotype of live-attenuated respiratory virus vaccine candidates following replication in vivo is an important consideration. Biologically significant genetic or phenotypic instability has been documented in some [31–33], but not all, live-attenuated respiratory virus vaccine candidates tested in humans [34]. Genetic and phenotypic instability can arise because missense-attenuating mutations in biologically derived viruses, typically, are based on single nucleotide substitutions that, given the high mutation rate of RNA viruses, can readily revert back to the wild type assignment. This problem can be greatly reduced by using codon substitutions that are chosen to differ by two or three nucleotides from codons that confer a wild type phenotype [18]. Codon substitution mutations were, therefore, introduced into site 460 or 948 of the HPIV2 L protein such that three nucleotide substitutions would be required to change the codon to one that specified the wild type amino acid. Interestingly, this identified mutants involving position 460 (rF460A and rF460P) that were more *ts* and *att* than the original rF460L mutant, and identified mutants involving position 948 (rY948A, rY948G, and rY948L) that were *ts* and *att*, whereas the original rY948H mutant was neither *ts* nor *att*. The stabilized mutations at sites 460 and 948 can now be combined into one virus to incrementally increase the level of attenuation and genetic stability of an HPIV2 vaccine candidate.

In the present study, a novel strategy was employed to generate a second type of stable attenuating mutation. This strategy involves the deletion of the amino acid sequence position in question. Deletions are considered more desirable mutations than single amino acid substitutions because they are expected to be more refractory to reversion. In some cases, attenuation specified by deletion mutations can be reversed either by second site suppressor mutations or by repair of the mutation by the viral enzymatic machinery [35–37], but, in general, they are more stable genetically than amino acid substitution mutations. Since HPIV2 adheres to the rule of six [6,9], a second, adjacent codon was deleted along with the codon of interest such that the total length of the genome was reduced by six nucleotides and maintained its hexamer spacing. The hexameric requirement likely confers additional stability against repair of the deletion, since a repaired virus will be viable only if all six deleted nucleotide positions are restored in a single step. Five deletion mutations were introduced into the HPIV2 cDNA, but only one deletion mutant was recovered. This low rate of success is not surprising, since the deletion of two amino acids is likely a more drastic change than substitution of a single amino acid. The rdel1724 mutant, which replicated efficiently in cell culture, was *ts* and highly restricted in replication in the upper and lower respiratory tract of hamsters, and, thus, rdel1724 represents a promising attenuating mutation. Because this attenuation phenotype is specified by a six-nucleotide deletion, it is expected to be quite stable, as already noted. However, this will require experimental confirmation in subsequent studies.

Previous findings and findings from the present study demonstrate that the importation of mutations from attenuated paramyxoviruses is a rapid and effective approach to the attenuation of wild type HPIV1, HPIV2, and HPIV3 [5,14,17]. It is particularly important in cases such as for HPIV1 and HPIV2, for which attenuated derivatives and attenuating mutations were not otherwise available. Recombinant HPIV2s bearing mutations identified in this study will be evaluated in African green monkeys, a non-human primate model for HPIV2 replication [38]. Mutations from the set of r460A, r460P, r948A, r948L, r948G, and rdel1724 that are confirmed to attenuate rHPIV2 for African green monkeys will then be used in combination to generate HPIV2 vaccine candidates that are attenuated by multiple mutations, each stabilized by one mechanism or another. The genetic and phenotypic stability of these vaccine candidates will be assessed following replication in vitro under conditions of serial passage with stepwise elevations in temperature (in the case of *ts* viruses) to determine whether they stably maintain the introduced mutations and the attenuation phenotype. These HPIV2 vaccine candidates will then be tested for immunogenicity and efficacy in African green monkeys and suitable candidates will be evaluated in clinical studies.

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Recombinant Respiratory Syncytial Virus That Does Not Express the NS1 or M2-2 Protein Is Highly Attenuated and Immunogenic in Chimpanzees

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Mutant recombinant respiratory syncytial viruses (RSV) which cannot express the NS1 and M2-2 proteins, designated rA2ΔNS1 and rA2ΔM2-2, respectively, were evaluated as live-attenuated RSV vaccines. The rA2ΔNS1 virus contains a large deletion that should have the advantageous property of genetic stability during replication in vitro and in vivo. In vitro, rA2ΔNS1 replicated approximately 10-fold less well than wild-type recombinant RSV (rA2), while rA2ΔM2-2 had delayed growth kinetics but reached a final titer similar to that of rA2. Each virus was administered to the respiratory tracts of RSV-seronegative chimpanzees to assess replication, immunogenicity, and protective efficacy. The rA2ΔNS1 and rA2ΔM2-2 viruses were 2,200- to 55,000-fold restricted in replication in the upper and lower respiratory tracts but induced a level of RSV-neutralizing antibody in serum that was only slightly reduced compared to the level induced by wild-type RSV. The replication of wild-type RSV in immunized chimpanzees after challenge was reduced more than 10,000-fold at each site. Importantly, rA2ΔNS1 and rA2ΔM2-2 were 10-fold more restricted in replication in the upper respiratory tract than was the *cpts*248/404 virus, a vaccine candidate that retained mild reactogenicity in the upper respiratory tracts of 1-month-old infants. Thus, either rA2ΔNS1 or rA2ΔM2-2 might be appropriately attenuated for this age group, which is the major target population for an RSV vaccine. In addition, these results show that neither NS1 nor M2-2 is essential for RSV replication in vivo, although each is important for efficient replication.

Respiratory syncytial virus (RSV) is the leading etiologic agent of serious pediatric viral bronchiolitis and pneumonia worldwide and is responsible for approximately 100,000 hospitalizations and 4,500 deaths among infants and children in the United States per annum (7, 14, 25). In addition, RSV infection can cause severe respiratory illness in the elderly (23) and in immunocompromised individuals (28). To date, an effective licensed vaccine for RSV is not available despite the pressing need for such an agent.

Since 1967, our laboratory has focused on developing a live-attenuated RSV vaccine for intranasal administration. By mimicking a natural infection, such a vaccine should stimulate both cellular and humoral immunity and would obviate the potentiated disease that was observed with certain nonreplicating or subunit vaccines (7, 16, 24, 27). The intranasal route also partially abrogates the immunosuppressive effects of maternal antibodies present in the sera of young infants and stimulates both local and systemic immunity (10).

A number of live-attenuated RSV vaccine candidates have been developed by biological or recombinant methods and evaluated in animals and humans (8, 15, 16, 29, 30, 32). The most promising biologically derived candidate, a cold-passaged (*cp*) temperature-sensitive (*ts*) virus called *cpts*248/404, was evaluated in RSV-naive 1- to 2-month-old infants and was found to be infectious, immunogenic, and protective against a second vaccine dose (33). However, some vaccinees experienced mild upper respiratory tract congestion, indicating that further attenuation is necessary. In addition, virus isolated late during the course of infection from a single vaccinee showed partial phenotypic reversion and loss of an attenuating mutation. Thus, our strategy to develop improved live-attenuated vaccine candidates has been (i) to use recombinant methods to combine attenuating mutations identified in a panel of biologically derived attenuated viruses including *cpts*248/404 and (ii) to develop new types of attenuating mutations by focusing on gene deletions which should be refractory to genetic reversion.

RSV is the prototype member of the *Pneumovirus* genus of the family *Paramyxoviridae*. Its genome is a single-stranded, negative-sense RNA of 15.2 kb that encodes 10 subgenomic mRNAs from which 11 proteins are translated. These proteins include the nucleocapsid N protein, phosphoprotein P, and large polymerase subunit L, which together comprise the minimal viral polymerase; fully processive transcription by the RSV polymerase requires the presence of the transcription antitermination factor M2-1 (6, 18, 19, 34). There are four envelope-associated proteins: the internal matrix (M) protein and three transmembrane surface proteins, namely, the attachment (G), fusion (F), and small hydrophobic (SH) proteins (7). Finally, RSV encodes two nonstructural proteins, NS1 and NS2, and also the M2-2 protein, whose status as structural or nonstructural is unknown. NS1 and M2-2 appear to have roles in RNA synthesis.

We previously described a reverse-genetics system for producing recombinant subgroup A RSV (rRSV) by coexpression of antigenomic RNA and the N, P, L, and M2-1 proteins from cotransfected plasmids (5). One application of this system has been to identify viral genes that can be deleted or silenced without ablating replication in vitro but are still necessary for

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virus replication in vivo (4, 26). Deletion of the SH gene resulted in a virus, designated rA2ΔSH, that replicated in vitro with an efficiency equal to or slightly better than that of wild-type rRSV (rA2) and which was moderately attenuated in mice and chimpanzees (4, 29). rRSV from which the NS2 gene was deleted, designated rA2ΔNS2, exhibited reduced growth kinetics and a reduced yield of infectious virus in vitro and was markedly attenuated in mice and chimpanzees (26, 29). Similar in vitro properties were noted for a recombinant bovine RSV from which the NS2 gene was deleted (2). These two deletion mutations are now being incorporated into recombinant live-attenuated vaccine candidates for clinical evaluation.

More recently, the M2-2 open reading frame was silenced in rRSV (rA2ΔM2-2, previously designated rA2-K5) by mutating each of the three potential translational initiation codons and inserting a translation termination codon in each of the three reading frames (1). A second research group made a comparable virus in which M2-2 was silenced by deletion of most of its open reading frame, which resulted in a virus that appeared to be phenotypically similar to rA2ΔM2-2 (20). The rA2ΔM2-2 virus exhibited increased plaque size, reduced growth kinetics (though the final titer was similar to that of the wild type), and a partial shift in RNA synthesis from RNA replication to transcription (1). Thus, the M2-2 protein appears to be a regulatory protein that negatively regulates transcription and positively regulates RNA replication. In addition, an rRSV was constructed from which the NS1 gene was deleted by the removal of nucleotides 122 to 630 in the antigenomic cDNA, resulting in the joining of the upstream nontranslated region of NS1 to the translational initiation codon of NS2. This virus, designated rA2ΔNS1, exhibited reduced RNA replication, plaque size, and growth kinetics and an approximately 10-fold lower yield of infectious virus in vitro (M. N. Teng and P. L. Collins, submitted for publication). Other paramyxoviruses encode proteins, such as the V protein of Sendai virus, that are not essential for replication in vitro. However, ablation of expression of V by recombinant Sendai virus results in attenuation in vivo (22). It was suggested that this protein functioned to antagonize some aspect of the mouse's innate immune system. More recently, the V protein of simian virus 5 was shown to block signalling for both type I and type II interferon responses (13). Any of the RSV "accessory" proteins, including the NS1, NS2, M2-2, SH, and G proteins, are candidates for antagonizing host immune mechanisms.

In the present study, we evaluated the rA2ΔM2-2 and rA2ΔNS1 viruses for replication, immunogenicity, and protective efficacy in the upper and lower respiratory tracts of chimpanzees, the only experimental animal in which RSV replication and virulence approaches that observed in humans. The rA2ΔM2-2 and rA2ΔNS1 viruses described above were constructed in the original version of the antigenomic cDNA described by Collins et al. (5). All recombinant viruses that have been constructed for vaccine purposes in our laboratory contain two types of modification to this background: (i) the introduction of a set of six translationally silent restriction markers in the L gene, called the sites mutations, and (ii) two amino acid substitutions in the F protein, called the HEK mutations, which make the recombinant virus identical at the amino acid level to the wild-type RSV A2 parent from which the *cpts*248/404 series of biological vaccine candidates was derived (21, 30). These mutations were shown to be phenotypically silent in chimpanzees (32). The rA2ΔNS1 virus used in this study was reconstructed in a sites-HEK background, in preparation for clinical evaluation, whereas the rA2ΔM2-2 virus is in the original genetic background, a difference that is not relevant for the present study (1, 30).

The rA2ΔNS1 and rA2ΔM2-2 viruses were administered individually to juvenile RSV-seronegative chimpanzees by combined intranasal and intratracheal inoculation, as described previously (11). Since both viruses were attenuated in vitro, we chose to inoculate the animals with 10^5 PFU per ml per site, which is a 10-fold higher concentration than that typically used to inoculate chimpanzees. To monitor virus replication in the upper and lower respiratory tracts, respectively, nasopharyngeal swabs and tracheal lavage samples were collected at intervals over 10 days postinfection and subsequently were assayed for virus titer. The mean peak virus titer was determined for each group (Table 1). The chimpanzees were monitored daily for rhinorrhea, a symptom of upper respiratory tract illness, and the mean peak score was determined for each group (Table 1). Due to the limited availability of RSV-seronegative chimpanzees, the number of animals per group was small, making it necessary to include controls from previous studies in which we had evaluated biologically derived RSV strain A2 (wild-type RSV A2), rA2, rA2ΔSH, rA2ΔNS2, and a recombinant version of the above-mentioned *cpts*248/404 vaccine candidate (rA2cp248/404) (Table 1).

Levels of replication of rA2ΔNS1 and rA2ΔM2-2 were reduced more than 2,200-fold and more than 2,800-fold, respectively, in the upper respiratory tract compared to that of rA2 (Table 1). Shedding of rA2ΔNS1 or rA2ΔM2-2 was detected sporadically and at a low level beginning 2 to 7 days postinfection, and each animal shed virus over a period of 3 to 8 days (data not shown). Thus, the recovered virus was not carried over from the initial inoculum but represented replication near the level of detection over a period of several days. In the lower respiratory tract, the level of replication of rA2ΔNS1 was reduced more than 17,000-fold compared to that of rA2, while rA2ΔM2-2 was undetectable at all time points (greater than 55,000-fold reduction). It is important to note that the dose of rA2ΔNS1 and rA2ΔM2-2 used was 10-fold greater than that of rA2. Furthermore, both viruses were more attenuated than rA2cp248/404, which was given at the same dose, particularly in the case of rA2ΔM2-2, which was not recovered from the lungs of infected chimps. In addition, both rA2ΔNS1 and rA2ΔM2-2 were unusual in being equally restricted in the upper and lower respiratory tracts. In the upper respiratory tract, each virus was approximately 10-fold more restricted than *cpts*248/404 and 175-fold more restricted than rA2ΔNS2. Since upper respiratory tract congestion was observed during clinical evaluation of the *cpts*248/404 virus in 1- to 2-month-old infants (33) and since infants of that age are obligate nose breathers, mutations that confer a level of restriction of replication in the upper respiratory tract greater than that of *cpts*248/404 would be desirable for inclusion in a live-attenuated vaccine virus. Animals receiving rA2ΔNS1 or rA2ΔM2-2 had slightly more rhinorrhea than those infected with rA2cp248/404, though still less than that of animals infected with a 10-fold smaller dose of rA2. While it is possible that the absence of NS1 or M2-2 resulted in a virus that retained a moderate level of virulence but replicated poorly, we think that this possibility is unlikely. Our experience is that quantitation of rhinorrhea and the comparison of such values from different studies performed at different times can be somewhat subjective and hence not completely reproducible. We anticipate that further evaluation, including clinical studies, will show that the amount of residual virulence associated with rA2ΔNS1 and rA2ΔM2-2 will reflect their greatly reduced replication.

Despite the highly restricted replication of these viruses, immunization with either rA2ΔNS1 or rA2ΔM2-2 induced a level of RSV-neutralizing antibody in serum that was within threefold of that induced by rA2cp248/404 (Table 1). Further-

TABLE 1. rA2ΔNS1 and rA2ΔM2-2 are highly attenuated in both the upper and lower respiratory tracts of chimpanzees but are highly immunogenic

Virus used to infect chimpanzees[a]	No. of animals	Dose[b] (per site, $\log_{10}$ PFU)	Mean peak virus titer[c] ($\log_{10}$ PFU/ml) ± SE (Duncan grouping)		Mean peak rhinorrhea score[d] (range, 0–4)	Mean neutralizing antibody titer in serum[e] (reciprocal $\log_2$)	
			Nasopharyngeal swab	Tracheal lavage		Day 0	Day 28
Wild-type RSV A2[f]	2	4.0	5.0 ± 0.35 (A)	5.5 ± 0.40 (A)	3.0	<3.3	11.2
rA2[g]	2	4.0	4.9 ± 0.15 (A)	5.4 ± 0.05 (A)	2.5	<3.3	10.5
rA2ΔSH[g]	3	4.0	4.6 ± 0.10 (A)	3.8 ± 0.31 (B)	1.0	<3.3	10.2
rA2ΔNS2[g]	4	4.0	3.8 ± 0.41 (B)	1.4 ± 0.29 (C)	1.0	3.4	10.6
rA2cp248/404[g]	4	5.0	2.5 ± 0.25 (C)	1.4 ± 0.37 (C)	0.8	3.4	10.6
rA2ΔNS1	4	5.0	1.6 ± 0.12 (D)	1.2 ± 0.43 (C)	2.0	<3.3	9.8
rA2ΔM2-2	4	5.0	1.5 ± 0.09 (D)	<0.7	1.8	<3.3	9.1

[a] All recombinant-derived viruses contain the sites and HEK mutations (see the text), except for rA2ΔM2-2.
[b] Chimpanzees were inoculated by the intranasal and intratracheal routes with the indicated amount of virus in a 1-ml inoculum per site.
[c] Nasopharyngeal swab samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10. Mean peak titers were calculated and assigned to statistically similar groups by Duncan's multiple-range test ($\alpha = 0.05$). Means in each column with different letters are significantly different.
[d] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity. Scores indicate severe (4), moderate (3), mild (2), trace (1), or no (0) rhinorrhea. Shown are the mean peak scores.
[e] Serum RSV-neutralizing antibody titers were determined by a complement-enhanced 60% plaque reduction assay using wild-type RSV A2 and HEp-2 cell monolayer cultures incubated at 37°C. RSV-seronegative chimpanzee serum used as a negative control had a neutralizing antibody titer of <3.3 $\log_2$ reciprocal. Adult human serum used as a positive control had a neutralizing antibody titer of 11.4 $\log_2$ reciprocal.
[f] Historic controls from the study of Crowe et al. (10).
[g] Data from the study of Whitehead et al. (29).

more, animals previously infected with either rA2ΔNS1 or rA2ΔM2-2 were highly resistant to the replication of wild-type RSV administered intranasally and intratracheally 56 days postimmunization (Table 2). The levels of protection in both cases were similar in the upper respiratory tract and somewhat lower in the lower respiratory tract than that seen with *cpts*248/404, both in mean peak titer and in mean days of shedding.

The challenge in developing a live-attenuated RSV vaccine is to eliminate residual virulence without compromising immunogenicity. Observations to date indicate that the severity of RSV disease is closely related to the level of RSV replication in the respiratory tract. It is possible that one or more attenuating mutations that reduce virulence through another mechanism will be identified; indeed, it was hoped that deletion of one or more of the nonessential RSV proteins, such as those described in the present paper, might reveal such a virulence factor. However, a factor of this nature has not yet been identified for RSV. Thus, the present method for attenuating RSV is to reduce its level of replication, which unfortunately can reduce its immunogenicity due to the reduced production of antigen. The attenuating mutations that we have identified to date include (i) a set of five amino acid substitutions in the N, F, and L proteins that were identified in *cp*RSV and that confer attenuation in chimpanzees and humans (9, 16, 32); (ii) a series of amino acid substitutions in the L protein and a nucleotide substitution in the gene-start signal of the M2 gene, which were identified in biologically derived *ts* derivatives of *cp* RSV and which each confer the *ts* and attenuation phenotypes (12, 15, 21, 30); and (iii) deletion of individual or combinations of RSV genes such as the SH and NS2 genes (4, 26). Bovine RSV genes have also been used to confer attenuation based on host range restriction (3). Here, we add two additional knock-

TABLE 2. Infection of chimpanzees with rA2ΔNS1 or rA2ΔM2-2 induced significant protection against subsequent challenge with wild-type RSV A2 in the upper and lower respiratory tracts

Immunizing virus	Inoculum dose[a] ($\log_{10}$ PFU/ml)	No. of animals	Replication of RSV challenge virus at the indicated site[b]				Mean peak rhinorrhea score
			Nasopharynx		Trachea		
			Mean no. of days of shedding ± SE	Mean peak titer[c] ± SE	Mean no. of days of shedding ± SE	Mean peak titer ± SE	
rA2ΔNS1	5.0	4	2.8 ± 0.75	1.7 ± 0.46	1.0 ± 0.41	1.8 ± 0.73	1.0
rA2ΔM2-2	5.0	4	3.5 ± 0.87	2.3 ± 0.71	1.0 ± 0.71	1.7 ± 0.63	1.0
rA2ΔNS2[d]	4.0	4	ND	1.9 ± 0.30	ND	2.2 ± 0.77	1.0
*cpts*248/404[e]	4.7	2	3.5 ± 0.50	2.3 ± 0.25	0	<0.7	1.0
None[e]		2	8.5 ± 0.50	5.0 ± 0.35	6.0 ± 1.0	4.8 ± 0.30	3.0

[a] Each virus was initially administered at the indicated dose in a 1.0-ml inoculum given intranasally and intratracheally.
[b] On day 56, chimpanzees were challenged with wild-type RSV A2 administered at a dose of 10^4 PFU/ml in a 1.0-ml inoculum given intranasally and intratracheally. Nasopharyngeal swab samples were collected daily for 12 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 12. ND, not determined.
[c] Mean peak titers ($\log_{10}$ PFU/ml) were calculated by using the peak virus titer achieved in each animal.
[d] Data from the study of Whitehead et al. (29).
[e] Historic control animals from the study of Crowe et al. (10) were used.

out mutations to the list, namely, the deletion of NS1 and the silencing of the M2-2 open reading frame.

Among the mutant viruses shown in Table 1, the order of increasing attenuation in seronegative juvenile chimpanzees was rA2ΔSH < rA2ΔNS2 < rA2cp248/404 < rA2ΔNS1 < rA2ΔM2-2. All viruses provided similar, high levels of protection against challenge with wild-type RSV (Table 2). Thus, rA2ΔNS1 and rA2ΔM2-2 each have the desired property of being slightly more attenuated than rA2cp248/404, the recombinant version of *cpts*248/404, which was slightly too reactogenic in RSV-naive 1- to 2-month-old infants, as mentioned above (33). The finding that rA2ΔM2-2 is slightly more attenuated than rA2ΔNS1 increases the chances that one of these viruses will have an optimal level of attenuation. The seronegative juvenile chimpanzee is somewhat less permissive to RSV replication and disease than is the RSV-naive human infant. Thus, whether rA2ΔNS1, rA2ΔM2-2, or both have an appropriate level of attenuation can be determined only by clinical trials with the target vaccine population, 1- to 2-month-old infants.

Deletion mutants should be extremely stable both in vitro and in vivo, thus making them attractive candidates for vaccine development. This property might be important in light of the finding that one infant who had been vaccinated with *cpts*248/404 shed virus that exhibited a partial reversion (33). A low level of genetic instability in an RSV vaccine likely would not be a problem in normal individuals, particularly considering the high prevalence of fully virulent wild-type RSV. However, vaccine virus might have prolonged replication in immunocompromised individuals. Thus, it would be desirable to engineer a recombinant vaccine virus to contain attenuating mutations that cannot revert.

Although the major target for an RSV vaccine is the 1- to 2-month-old infant, a second target is the elderly. The *cpts*248/404 vaccine candidate, which was insufficiently attenuated in the RSV-naive infant, was found to be overattenuated in the RSV-experienced adult (17). Thus, a live-attenuated vaccine for RSV-naive infants will need to be more attenuated than one for use in adults. Since the rA2ΔNS1 and rA2ΔM2-2 viruses are similar to *cpts*248/404 in their levels of replication, they likely will be too attenuated to be useful as an adult vaccine. However, each virus is appropriate for further evaluation as a pediatric RSV vaccine, either as currently constructed or with the inclusion of a single or a combination of additional attenuating mutations. It should be noted that if either candidate vaccine proves satisfactory, a partner subgroup B candidate can be rapidly generated by replacing the F and G glycoproteins (31).

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Mortality Associated With Influenza and Respiratory Syncytial Virus in the United States

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Context Influenza and respiratory syncytial virus (RSV) cause substantial morbidity and mortality. Statistical methods used to estimate deaths in the United States attributable to influenza have not accounted for RSV circulation.

Objective To develop a statistical model using national mortality and viral surveillance data to estimate annual influenza- and RSV-associated deaths in the United States, by age group, virus, and influenza type and subtype.

Design, Setting, and Population Age-specific Poisson regression models using national viral surveillance data for the 1976-1977 through 1998-1999 seasons were used to estimate influenza-associated deaths. Influenza- and RSV-associated deaths were simultaneously estimated for the 1990-1991 through 1998-1999 seasons.

Main Outcome Measures Attributable deaths for 3 categories: underlying pneumonia and influenza, underlying respiratory and circulatory, and all causes.

Results Annual estimates of influenza-associated deaths increased significantly between the 1976-1977 and 1998-1999 seasons for all 3 death categories ($P<.001$ for each category). For the 1990-1991 through 1998-1999 seasons, the greatest mean numbers of deaths were associated with influenza A(H3N2) viruses, followed by RSV, influenza B, and influenza A(H1N1). Influenza viruses and RSV, respectively, were associated with annual means (SD) of 8097 (3084) and 2707 (196) underlying pneumonia and influenza deaths, 36155 (11055) and 11321 (668) underlying respiratory and circulatory deaths, and 51203 (15081) and 17358 (1086) all-cause deaths. For underlying respiratory and circulatory deaths, 90% of influenza- and 78% of RSV-associated deaths occurred among persons aged 65 years or older. Influenza was associated with more deaths than RSV in all age groups except for children younger than 1 year. On average, influenza was associated with 3 times as many deaths as RSV.

Conclusions Mortality associated with both influenza and RSV circulation disproportionately affects elderly persons. Influenza deaths have increased substantially in the last 2 decades, in part because of aging of the population, underscoring the need for better prevention measures, including more effective vaccines and vaccination programs for elderly persons.

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INFLUENZA INFECTIONS RESULT IN substantial morbidity and mortality nearly every year[1,2] and estimates of this burden have played a pivotal role in formulating influenza vaccination policy in the United States.[3] However, numbers of deaths attributable to influenza are difficult to estimate directly because influenza infections typically are not confirmed virologically or specified on hospital discharge forms or death certificates. In addition, many influenza-associated deaths occur from secondary complications when influenza viruses are no longer detectable.[4,5] Nonetheless, wintertime influenza epidemics have been shown to be associated with increased hospitalizations and mortality for many diagnoses, including congestive heart failure, chronic obstructive pulmonary disease, pneumonia, and bacterial superinfections.[6-9]

Respiratory syncytial virus (RSV) epidemics often overlap with influenza epidemics,[8,10] and RSV infections have been associated with substantial morbidity and mortality in young children and more recently in older adults.[10-14] Like influenza, RSV infections can precipitate both cardiac and pulmonary complications.[15-17] Respiratory syncytial virus infections are rarely diagnosed in

For editorial comment see p 227.

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adults, in part because available rapid antigen-detection tests are insensitive in adults and few tests for RSV are requested for this age group by medical practitioners.[16,18] It is likely that some deaths previously attributed to influenza are actually associated with RSV infection.[13,14,19]

In this study, we provide age-specific estimates of deaths attributable to influenza, by virus type and subtype, and to RSV using Poisson regression models that incorporates national respiratory viral surveillance data. Recent deliberations of the Advisory Committee on Immunization Practices (ACIP) regarding influenza vaccination recommendations[3] guided our choice of age groups for these analyses.

METHODS

Definition of Respiratory Season

Influenza and RSV typically circulate during winter months and across calendar years. Therefore, we defined each annual respiratory season as the period from July 1 through June 30 of the following year.

National Viral Surveillance Data

In the United States, laboratory-based surveillance for influenza viruses is conducted from October through mid-May (calendar week 40 through week 20). For the influenza virus surveillance periods from the 1976-1977 through 1998-1999 seasons, we obtained numbers of influenza virus isolates reported weekly by 50 to 75 World Health Organization collaborating virology laboratories in the United States to the Influenza Branch of the Centers for Disease Control and Prevention (CDC). The collaborating laboratories provided weekly numbers of total respiratory specimens tested for influenza and positive-influenza isolates by virus type and subtype[20] (TABLE 1).

Weekly RSV data were obtained from the National Respiratory and Enteric Virus Surveillance System for the 1990-1991 through 1998-1999 seasons. During this period, 63 to 72 clinical and public health laboratories in 44 states reported to CDC weekly numbers of specimens tested for RSV by antigen-detection and viral-isolation methods and numbers of positive results.[21] We used the results of both antigen-detection and isolation tests to determine the circulation pattern of RSV. The weekly percentages of specimens that tested positive for both influenza and RSV were used in estimating the association of virus circulation with weekly deaths in the United States (Table 1).

Mortality Data and Outcomes

National mortality data were obtained from the National Center for Health Statistics (NCHS).[22] Deaths were categorized using the *International Classification of Diseases, Ninth Revision (ICD-9)* codes for NCHS mortality data obtained from 1976 through 1998.[23]

Table 1. Annual Respiratory Virus Surveillance Data for the 1976-1977 Through 1998-1999 Seasons

	No. of Influenza Specimens					No. of RSV Specimens	
Season	Specimens Tested	A(H1N1) Positive Isolates	A(H3N2) Positive Isolates	B Positive Isolates	Total Positive Isolates	Specimens Tested	Total Positive Tests
1976-1977	17 600	3	212	633	848	NA	NA
1977-1978	18 727	311	1617	5	1933	NA	NA
1978-1979	13 275	1140	1	21	1162	NA	NA
1979-1980	15 195	20	17	1298	1335	NA	NA
1980-1981	16 128	315	1125	1	1441	NA	NA
1981-1982	14 804	143	0	461	604	NA	NA
1982-1983	16 929	165	1263	160	1588	NA	NA
1983-1984	16 111	1059	79	937	2075	NA	NA
1984-1985	15 355	2	1977	53	2032	NA	NA
1985-1986	20 234	2	554	1789	2345	NA	NA
1986-1987	22 056	2206	5	11	2222	NA	NA
1987-1988	26 258	167	1776	354	2297	NA	NA
1988-1989	29 357	2234	359	2530	5123	NA	NA
1989-1990	29 956	46	3342	13	3401	NA	NA
1990-1991	32 420	179	271	2732	3182	67 374	11 449
1991-1992	38 557	1055	4854	47	5956	100 867	18 586
1992-1993	36 233	132	1126	3081	4339	98 203	14 335
1993-1994	35 597	22	4193	35	4250	104 028	18 047
1994-1995	38 705	62	2819	1005	3886	107 528	17 445
1995-1996	37 612	2357	1650	716	4723	111 318	19 745
1996-1997	39 183	0	5047	1449	6496	117 814	17 370
1997-1998	46 413	14	7838	53	7905	133 648	19 589
1998-1999	52 505	50	6732	2286	9068	128 621	18 418
Mean (SD)	27 360 (11 551)	508 (779)	2037 (2277)	855 (998)	3400 (2290)	107 711 (19 374)	17 220 (2685)

Abbreviations: NA, not applicable (reporting period began in 1990-1991); RSV, respiratory syncytial virus.

The *International Classification of Diseases, 10th Revision (ICD-10)* was used for classifying NCHS mortality data obtained for 1999.[24] Analyses were based on the underlying cause-of-death because it represents the disease or injury that initiated the chain of morbid events that led directly to the death.[25] The change from *ICD-9* to *ICD-10* in January 1999 resulted in a 30% decrease in the number of coded underlying pneumonia deaths.[26] Therefore, all analyses of underlying pneumonia and influenza deaths were carried out using data collected through the 1997-1998 season, which limited the analyses to *ICD-9* coded deaths.

The 3 death categories modeled were underlying pneumonia and influenza deaths (*ICD-9* codes 480-487), underlying respiratory and circulatory deaths (*ICD-9* codes 390-519 and *ICD-10* codes I00-I99, J00-J99), and all-cause deaths (all *ICD* codes). Underlying pneumonia and influenza deaths exclude some deaths, such as those related to exacerbations of underlying cardiac and pulmonary conditions, which are associated with both influenza and RSV infections.[6,18] Influenza-associated all-cause death estimates have been previously used to represent the full spectrum of deaths associated with influenza infections.[1,2] However, these estimates include deaths such as those caused by fires and motor vehicle crashes, which are not directly associated with respiratory viral infections. Therefore, we also modeled a third category of deaths, underlying respiratory and circulatory deaths (which includes pneumonia and influenza deaths), to provide an estimate of deaths that was more directly associated with viral respiratory infections. These estimates would be expected to be more sensitive than estimates using underlying pneumonia and influenza deaths and more specific than estimates using all-cause deaths.

Statistical Analyses

For the influenza model, we developed an age-specific Poisson regression model that used weekly influenza circulation data. Deaths were stratified into the following 5 age groups: younger than 1 year, 1 to 4 years, 5 to 49 years, 50 to 64 years, and 65 years or older. Influenza-associated deaths were estimated for influenza A(H1N1), A(H3N2), and B viruses. The viral circulation terms represented percentages of specimens testing positive for each of the 3 influenza virus types and subtypes during a particular week. Estimates of the weekly age-specific population size were used to account for changes in population trends over time. The US population estimates by age group were obtained from the US Census Bureau.[27]

For the influenza and RSV model, we used a model identical to the influenza model except it included an additional coefficient for RSV viral circulation. The full model was written as follows:

$$Y = \alpha \exp\{\beta_0 + \beta_1[t] + \beta_2[t^2] + \beta_3[\sin(2\pi/52)] + \beta_4[\cos(2\pi/52)] + \beta_5[A(H1N1)] + \beta_6[A(H3N2)] + \beta_7[B] + \beta_8[RSV]\}$$

where Y represents the number of deaths during a particular week for a specific age group, α is the offset term and is equal to the log of the age-specific population size, β_0 represents the intercept, β_1 accounts for the linear time trend, β_2 accounts for nonlinear time trends, β_3 and β_4 account for seasonal changes in deaths, and β_5 through β_8 represent coefficients associated with the percentage of specimens testing positive for a given week.

We fit the influenza model to national influenza surveillance data available from the 1976-1977 through 1998-1999 seasons. The influenza and RSV model was fit to data available from the 1990-1991 through 1998-1999 seasons, when both weekly influenza and RSV data were available (PROC GENMOD, SAS, version 8.2; SAS Institute Inc, Cary, NC).

RESULTS

Annual Influenza and RSV Laboratory Surveillance

National influenza and RSV surveillance data are summarized in Table 1. Influenza isolate data were available for the 1976-1977 through 1998-1999 seasons. A mean of 27 360 specimens (range, 13 275-52 505) was tested for influenza viruses during each of the influenza surveillance periods (October through May). During weeks that testing for influenza occurred, an average of 12% of specimens tested positive for influenza. Influenza A(H1N1), A(H3N2), and B viruses, respectively, comprised 15%, 60%, and 25% of the positive influenza isolates. From the 1990-1991 through 1997-1998 season, the annual mean number of specimens tested for RSV was 107 711 (range, 67 374-133 648) with an average of 17 220 specimens (16%) testing positive each season for RSV.

Annual US Deaths by Underlying Diagnosis

From the 1976-1977 through 1998-1999 seasons, there was an annual mean of 69 140 (range, 47 133-90 895) underlying pneumonia and influenza deaths, 1 135 724 (range, 1 069 560-1 203 728) underlying respiratory and circulatory deaths, and 2 126 740 (range, 1 879 039-2 407 494) all-cause deaths. The numbers of deaths in each of these categories increased linearly during this period. From the 1976-1977 through 1997-1998 seasons, underlying pneumonia and influenza deaths increased by 83%, substantially more than underlying respiratory and circulatory deaths or all-cause deaths (11% and 28%, respectively). From the 1990-1991 through 1998-1999 seasons, there was an annual mean of 82 239 (range, 74 872-90 895) underlying pneumonia and influenza deaths, 1 158 964 (range, 1 098 086-1 203 728) underlying respiratory and circulatory deaths, and 2 277 268 (range, 2 135 976-2 407 494) all-cause deaths.

Annual Influenza-Associated Deaths From the 1976-1977 Through 1998-1999 Seasons Using the Influenza Model

The mean annual estimates of underlying pneumonia and influenza deaths, underlying respiratory and circula-

tory deaths, and all-cause deaths associated with influenza were 5977, 25 420, and 34 470 (TABLE 2). Each of these 3 estimates increased significantly during the study period ($P<.001$ for trend for all 3 death categories).

Annual Influenza- and RSV-Associated Deaths From the 1990-1991 Through 1998-1999 Seasons Using the Influenza and RSV Model

For underlying pneumonia and influenza deaths, we estimated an annual mean of 8097 (SD, 3084; range, 3515-13 033) influenza-associated deaths, representing 9.8% (8097/82 239) of these deaths (TABLE 3). Influenza A(H1N1), A(H3N2), and B viruses were associated with annual means of 381 (SD, 617; range, 0-1742), 6613 (SD, 3928; range, 944-12 941), and 1103 (SD, 1030; range, 53-2619) deaths, respectively. Respiratory syncytial virus was associated with an annual mean of 2707 (SD, 196; range 2336-2880) deaths or 3.3% (2707/82 239) of all such deaths. The year-to-year variation in influenza-associated deaths was higher than the year-to-year variation in RSV-associated deaths.

For underlying respiratory and circulatory deaths, we estimated an annual mean of 36 155 (SD, 11 055; range, 17 056-51 296) influenza-associated deaths, representing 3.1% (36 155/1 158 964) of these deaths. Influenza A(H1N1), A(H3N2), and B viruses were associated with annual means of 1960 (SD, 3372; range, 0-10 080), 28 940 (SD, 14 848; range, 4435-50 855), and 5255 (SD, 4513; range, 253-12 067) deaths, respectively. Respiratory syncytial virus was associated with an annual mean of 11 321 (SD, 668; range, 10 047-12 385) deaths or 1.0% (11 321/1 158 964) of all such deaths.

For all-cause deaths, we estimated an annual mean of 51 203 (SD, 15 081; range, 25 570-71 416) influenza-associated deaths, representing 2.2% (51 203/2 277 268) of these deaths. Influenza A(H1N1), A(H3N2), and B viruses were associated with annual means of 2836 (SD, 4909; range, 0-14 727), 40 017 (SD, 20 656; range, 6033-70 701), and 8349 (SD, 7105; range, 404-19 030) deaths, respectively. Respiratory syncytial virus was associated with an annual mean of 17 358 (SD, 1086; range, 15 464-19 262) deaths or 0.8% (17 358/2 277 268) of all such deaths. Influenza-associated deaths again showed higher year-to-year variability than did RSV-associated deaths.

Table 2. Estimated Annual Influenza-Associated Deaths for the 1976-1977 Through 1998-1999 Seasons Using the Influenza Model

Season	Predominant Influenza Type and Subtype	No. of Deaths: Underlying Pneumonia and Influenza	No. of Deaths: Underlying Respiratory and Circulatory	No. of Deaths: All-Cause
1976-1977	B/A(H3N2)	2265	13 294	16 263
1977-1978	A(H3N2)/A(H1N1)	4449	26 829	32 172
1978-1979	A(H1N1)	1008	4692	7608
1979-1980	B	2359	10 605	13 832
1980-1981	A(H3N2)/A(H1N1)	4068	22 338	27 729
1981-1982	B-A(H1N1)	1260	5524	7612
1982-1983	A(H3N2)	5743	29 106	36 701
1983-1984	A(H1N1)/B	3437	14 051	19 923
1984-1985	A(H3N2)	8644	40 457	50 789
1985-1986	B/A(H3N2)	4649	18 923	24 994
1986-1987	A(H1N1)	1257	4650	8144
1987-1988	A(H3N2)	5307	23 376	30 755
1988-1989	B/A(H1N1)	5149	18 115	26 408
1989-1990	A(H3N2)	8254	34 602	45 493
1990-1991	B	4448	16 036	22 732
1991-1992	A(H3N2)/A(H1N1)	9449	37 159	50 563
1992-1993	B/A(H3N2)	7366	26 816	37 729
1993-1994	A(H3N2)	9717	37 367	50 729
1994-1995	A(H3N2)/B	7791	29 476	40 950
1995-1996	A(H1N1)/A(H3N2)	6560	24 562	36 280
1996-1997	A(H3N2)/B	13 674	48 726	68 328
1997-1998	A(H3N2)	14 628	52 148	72 399
1998-1999*	A(H3N2)/B	NA	45 817	64 684
Mean (SD)		5977 (3727)	25 420 (13 898)	34 470 (18 988)

Abbreviation: NA, not applicable.
*Pneumonia and influenza estimates are based on the 1976-1977 through 1997-1998 seasons.

Age-Specific Annual Influenza- and RSV-Associated Deaths From the 1990-1991 Through 1998-1999 Seasons Using the Influenza and RSV Model

In children younger than 1 year, RSV was associated with annual means of 124 underlying pneumonia and influenza deaths, 211 underlying respiratory and circulatory deaths, and 214 all-cause deaths (TABLE 4). In this age group, influenza viruses were associated with annual means of 13 underlying pneumonia and influenza deaths, 26 underlying respiratory and circulatory deaths, and 88 all-cause deaths. There were more influenza-associated deaths relative to RSV-associated deaths among children aged 1 to 4 years for all 3 death categories.

Among underlying pneumonia and influenza deaths, 90% (7326/8097) of influenza-associated deaths and 88% (2388/2707) of RSV-associated deaths occurred among persons aged 65 years or older. For underlying respiratory and circulatory deaths, 90% (32 651/36 155) of influenza-associated deaths and 78% (8811/11 321) of RSV-associated deaths occurred among persons aged 65 years or older. For all-cause deaths, 43 979 and 9812 all-cause deaths were attributable to influenza and RSV, respectively.

Age-Specific Mortality Rates

Annual mean influenza-associated mortality rates for underlying pneumonia and influenza deaths, underlying respiratory and circulatory deaths, and all-cause deaths were 3.1, 13.8, and 19.6 per 100 000 person-years, respectively (TABLE 5). Similarly, annual mean RSV-associated mortality rates for these death categories were 1.0, 4.3, and 6.6 per 100 000 person-years, respectively. The relative risks (RRs) and 95% confidence intervals (CIs) comparing influenza mortality rates with RSV mortality rates for the 3 death categories were 3.0 (95% CI, 2.9-3.1), 3.2 (95% CI, 3.1-3.3), and 2.9 (95% CI, 2.9-3.0), respectively.

Annual mean influenza-associated mortality rates for underlying pneumonia and influenza deaths in persons younger than 1 year, 1 to 4 years, 5 to 49 years, 50 to 64 years, and 65 years or older were 0.3, 0.2, 0.2, 1.3, and 22.1 deaths per 100 000 person-years, respectively. The RSV-associated mortality rates for underlying pneumonia and influenza deaths in persons younger than 1 year, 1 to 4 years, 5 to 49 years, 50 to 64 years, and 65 years or older were 3.1, 0.1, <0.1, 0.5, and 7.2 deaths per 100 000 person-years, respectively. For children younger than 1 year, the RR for RSV vs influenza mortality rates was 9.5 (95% CI, 5.4-16.9) for underlying pneumonia and influenza deaths and 8.1 (95% CI, 5.4-12.2) for underlying respiratory and circulatory deaths. For all-cause deaths, the RR among this age group was substantially lower (RR, 2.4; 95% CI, 1.9-3.1).

Age-Specific Mortality Rates Among Persons 65 Years or Older

Periseason influenza rate-difference models[10] were fit to the 1976-1977 through the 1998-1999 seasons and revealed substantial differences in relative influenza-attributable mortality rates among elderly persons. Persons aged 85 years or older were 32 times more likely to die of an influenza-associated underlying pneumonia and influenza death compared with persons aged 65 to 69 years (RR, 32.1; 95% CI, 31.3-32.9). Persons aged 85 years or older were 16 times more likely to die of an influenza-associated all-cause death compared with persons aged 65 to 69 years (RR, 14.8; 95% CI, 14.6-14.9). However, there were no statistically significant increases in any of the 5-year age-specific mortality rates from the 1976-1977 through the 1998-1999 seasons ($P>.05$ for all).

The number of persons aged 65 years or older increased substantially between the 1976-1977 and 1998-1999 seasons.[27] During the 1990s, the growth rate for the number of persons aged 50 to 64 years also increased substantially relative to the period from 1976 through 1990.[27]

COMMENT

Morbidity and mortality associated with seasonal epidemics of influenza in the United States have provided the impetus for public health policies and strategies to control influenza infections, particularly among specific target groups.[3] Mortality associated with in-

Table 3. Estimated Annual Influenza- and Respiratory Syncytial Virus–Associated Deaths for the 1990-1991 Through 1998-1999 Seasons Using the Influenza and RSV Model

Season	No. of Influenza Deaths				No. of Total RSV Deaths
	A(H1N1)	A(H3N2)	B	Total	
Underlying Pneumonia and Influenza Deaths					
1990-1991	226	944	2345	3515	2472
1991-1992	845	7904	73	8822	2858
1992-1993	142	3227	2619	5988	2336
1993-1994	20	8530	53	8603	2820
1994-1995	65	5710	995	6770	2781
1995-1996	1742	3816	964	6522	2880
1996-1997	0	9831	1691	11 522	2729
1997-1998	10	12 941	82	13 033	2778
1998-1999*	NA	NA	NA	NA	NA
Mean (SD)	381 (617)	6613 (3928)	1103 (1030)	8097 (3084)	2707 (196)
Underlying Respiratory and Circulatory Deaths					
1990-1991	1386	4435	11 235	17 056	11 156
1991-1992	4594	33 927	357	38 878	11 795
1992-1993	822	14 465	12 067	27 354	10 047
1993-1994	118	35 763	253	36 134	11 479
1994-1995	389	24 475	4473	29 337	11 797
1995-1996	10 080	16 895	4639	31 614	12 385
1996-1997	0	40 131	7803	47 934	11 105
1997-1998	47	50 855	394	51 296	10 806
1998-1999	203	39 514	6076	45 793	11 322
Mean (SD)	1960 (3372)	28 940 (14 848)	5255 (4513)	36 155 (11 055)	11 321 (668)
All-Cause Deaths					
1990-1991	1988	6033	17 549	25 570	16 947
1991-1992	6518	45 928	566	53 012	17 825
1992-1993	1190	19 892	19 030	40 112	15 464
1993-1994	173	48 923	404	49 500	17 581
1994-1995	572	33 767	7129	41 468	18 312
1995-1996	14 727	23 605	7509	45 841	19 262
1996-1997	0	55 937	12 609	68 546	17 100
1997-1998	66	70 701	649	71 416	16 461
1998-1999	293	55 367	9698	65 358	17 273
Mean (SD)	2836 (4909)	40 017 (20 656)	8349 (7105)	51 203 (15 081)	17 358 (1086)

Abbreviations: NA, not applicable; RSV, respiratory syncytial virus.
*Pneumonia and influenza estimates are based on the 1990-1991 through 1997-1998 seasons.

Table 4. Estimated Annual Age-Specific Influenza- and Respiratory Syncytial Virus–Associated Deaths for the 1990-1991 Through 1998-1999 Seasons

Age Group, y	No. of Influenza Deaths				No. of Total RSV Deaths
	A(H1N1)	A(H3N2)	B	Total	
Underlying Pneumonia and Influenza Deaths*					
<1	1	12	0	13	124
1-4	7	11	7	25	13
5-49	39	178	55	272	0
50-64	37	322	102	461	182
≥65	298	6089	939	7326	2388
Total	**382**	**6612**	**1103**	**8097**	**2707**
Underlying Respiratory and Circulatory Deaths					
<1	4	15	7	26	211
1-4	7	42	17	66	24
5-49	168	484	137	789	641
50-64	196	2121	306	2623	1634
≥65	1585	26278	4788	32651	8811
Total	**1960**	**28940**	**5255**	**36155**	**11321**
All-Cause Deaths					
<1	0	3	85	88	214
1-4	34	103	38	175	132
5-49	501	1685	383	2569	4464
50-64	348	3360	684	4392	2736
≥65	1954	34866	7159	43979	9812
Total	**2837**	**40017**	**8349**	**51203**	**17358**

Abbreviation: RSV, respiratory syncytial virus.
*Pneumonia and influenza estimates are based on the 1990-1991 through 1997-1998 seasons.

Table 5. Estimated Annual Influenza- and Respiratory Syncytial Virus–Associated Mortality Rates per 100 000 Person-Years for the 1990-1991 Through 1998-1999 Seasons

Age Group, y	Mortality Rate per 100 000 Person-Years	
	Influenza	RSV
Underlying Pneumonia and Influenza Deaths*		
<1	0.3	3.1
1-4	0.2	0.1
5-49	0.2	<.01
50-64	1.3	0.5
≥65	22.1	7.2
Total	3.1	1.0
Underlying Respiratory and Circulatory Deaths		
<1	0.6	5.3
1-4	0.4	0.2
5-49	0.5	0.4
50-64	7.5	4.7
≥65	98.3	26.5
Total	13.8	4.3
All-Cause Deaths		
<1	2.2	5.4
1-4	1.1	0.9
5-49	1.5	2.6
50-64	12.5	7.8
≥65	132.5	29.6
Total	19.6	6.6

Abbreviation: RSV, respiratory syncytial virus.
*Pneumonia and influenza estimates are based on the 1990-1991 through 1997-1998 seasons.

fluenza can vary dramatically by season and models developed to assess influenza-associated mortality date back to 1847.[28] These approaches have been feasible because well-defined peaks in deaths occur in association with influenza outbreaks in temperate countries. In the recent past, the CDC has used a linear regression model, applied to either complete national mortality data or more immediately available mortality surveillance data from 122 cities, to estimate annual deaths associated with influenza.[1,2] The influenza and RSV model presented in this study will be used to provide future estimates of influenza-associated mortality in the United States, because the model permits estimates of influenza subtype-specific mortality and also simultaneously estimates RSV-associated mortality.

Our results indicate that US influenza-associated deaths have increased substantially from the 1976-1977 through 1998-1999 seasons. We believe this is explained in part by the aging of the US population. Between 1976 and 1999, the number of persons aged 85 years or older doubled in the United States.[29] We found that persons in this age group were 16 times more likely to die of an influenza-associated all-cause death than persons aged 65 to 69 years during a period in which all-cause age-specific death rates have remained stable. Other studies have also shown that influenza-attributable mortality rates increased rapidly with age among persons aged 65 years or older.[30-32] For example, Nordin et al[32] found that persons aged 75 years or older were 3 to 9 times more likely to die from influenza infections than persons aged 65 to 74 years. Another important factor contributing to the increase in influenza-associated deaths during the 1990s was the predominance of influenza A(H3N2) viruses, the most virulent of the recently circulating influenza viruses. Influenza A(H3N2) viruses were 1 of the predominant strains in 8 of 9 seasons we analyzed during the 1990s.

The influenza and RSV model confirmed that influenza A(H3N2) viruses were associated with the highest attributable mortality rates, followed by RSV, influenza B, and influenza A(H1N1) viruses. The annual effect of RSV on mortality was relatively stable, although the numbers of deaths associated with influenza viruses varied substantially, depending on the predominant circulating virus type or subtype. In this study, RSV was the most common viral cause of death in children younger than 5 years, particularly in children younger than 1 year. However, RSV-associated mortality rates were higher in elderly persons and substantially more RSV-associated deaths occurred among elderly persons than among young children.

Determining the most appropriate death category for characterizing the burden of influenza on mortality is difficult. Pneumonia and influenza deaths are highly correlated with the circulation of influenza, and these estimates are useful for monitoring year-to-year trends and variability in the severity of influenza seasons. However, this death category underestimates the total burden of influenza because many deaths are

caused by other secondary complications of influenza (eg, congestive heart failure).[7] Traditionally, all-cause deaths have been used to estimate the total burden of influenza on mortality.[1,2] However, this death category is also not ideal because it includes deaths that are not causally linked with respiratory viral infections. Therefore, we analyzed underlying respiratory and circulatory deaths to provide a more specific estimate of the total burden of influenza and RSV on mortality. Our estimate of annual mean influenza-associated underlying respiratory and circulatory deaths was 36 155 (29% lower than the annual mean all-cause estimate).

The Poisson regression models used to estimate influenza- and RSV-associated deaths were more complex compared with previous influenza models, but the models also provided more specific estimates, including independent estimates of deaths associated with influenza and RSV. The model we used in this study is capable of incorporating additional factors that could not be included in the previous CDC model, such as temperature, that might independently influence winter season mortality. Therefore, we believe this new model represents a step forward in current efforts to better understand the burden of viral respiratory infections on mortality.

We applied the new model to age groups relevant to policy deliberations by ACIP regarding influenza-vaccination recommendations for persons younger than 5 years and persons 50 to 64 years.[3] Currently, ACIP recommends annual influenza vaccination for all persons aged 65 years or older. Because most influenza-associated deaths occur in persons aged 65 years or older, understanding age-specific effects within this age group is also of considerable interest. As a first step, we fit simple periseason rate difference models among those aged 65 years or older by 5-year age intervals and demonstrated increases with age in influenza-associated mortality. Future research will focus attention on ACIP discussions regarding age-specific vaccination policy.

We believe the results of this study have important policy implications. Deaths associated with viral respiratory infections have increased substantially during the past decade and it appears that they will continue to increase as the population continues to age. Increased numbers of patients with serious respiratory infections may further stress hospital systems that are already struggling to cope with wintertime surges in patient visits during influenza seasons. For example, during the 1997-1998 season, a severe influenza outbreak in Los Angeles County resulted in a dramatic increase in hospitalizations and the need to divert patients to other facilities. This problem may have been less severe if more bed capacity had been available.[33]

Vaccinating elderly persons will continue to be the primary strategy for preventing influenza-associated deaths. Studies directly comparing outcomes in vaccinated vs unvaccinated groups have shown that the currently available trivalent inactivated influenza vaccine is approximately 68% effective in preventing deaths from complications of influenza infections.[34-36] However, the effectiveness of influenza vaccines in preventing deaths among elderly persons with associated chronic conditions is significantly lower,[7,35] underscoring the need for influenza vaccines that are more immunogenic in elderly persons.[37,38] Recent studies have also raised the question of whether vaccinating young children against influenza may decrease transmission rates and thus decrease influenza-associated morbidity and mortality among elderly persons,[39,40] but the effectiveness of this approach remains uncertain.

Although the importance of RSV among young children is well recognized,[41,42] we found that more than 78% of RSV-associated underlying respiratory and circulatory deaths occured among persons aged 65 years or older. This finding highlights the need for an effective RSV vaccine in both young children and elderly persons.[12,16,18,43] A number of candidate RSV vaccines are being developed including vaccines based on cold-adapted live attenuated RSV strains[44,45] and subunit vaccines intended for use in RSV nonnaive populations.[46] Effective and safe vaccines for use among persons aged 65 years or older are needed to decrease deaths associated with RSV infection.

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Recombinant Respiratory Syncytial Virus (RSV) Bearing a Set of Mutations from Cold-Passaged RSV Is Attenuated in Chimpanzees

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A set of five missense mutations previously identified by nucleotide sequence analysis of subgroup A cold-passaged (*cp*) respiratory syncytial virus (RSV) has been introduced into a recombinant wild-type strain of RSV. This recombinant virus, designated rA2cp, appears to replicate less efficiently in the upper and lower respiratory tracts of seronegative chimpanzees than either biologically derived or recombinant wild-type RSV. Infection with rA2cp also resulted in significantly less rhinorrhea and cough than infection with wild-type RSV. These findings confirm the role of the *cp* mutations in attenuation of RSV and identify their usefulness for inclusion in future live attenuated recombinant RSV vaccine candidates.

Infection with respiratory syncytial virus (RSV) remains the most common cause of serious viral bronchiolitis and pneumonia in infants and children worldwide. The age distribution of serious RSV-associated illness is unusual among the respiratory tract pathogens in that disease occurs most commonly during the first several months of life, despite the presence of maternally transmitted serum neutralizing antibodies (5). Respiratory tract disease occurs less frequently and is generally less severe during reinfection with RSV than following first infection (5). Furthermore, the initial infection with RSV confers a significant degree of protection, and serum and local antibodies have been found to be mediators of immunity to RSV (5). The primary goal of immunization with an RSV vaccine is to prevent the severe lower respiratory tract disease associated with first and second infections with RSV. Live attenuated RSV vaccines represent promising vaccine candidates for this purpose since (i) they can efficiently immunize in the presence of passively acquired RSV antibodies which are present in the target population, the very young infant (9); (ii) they induce both serum immunoglobulin G and local immunoglobulin A antibodies; and (iii) they do not cause immune response-mediated disease enhancement like that seen following immunization with formalin-inactivated RSV (3).

Since early work indicated that attenuated mutants of poliovirus and measles virus could be selected by growth at low temperature (20, 21), a cold-passaged RSV (*cp*RSV) subgroup A candidate was produced from the A2 strain of RSV by 52 passages in bovine embryonic kidney tissue culture at progressively lower temperatures, the final and lowest temperature being 26°C (12). *cp*RSV was shown to be completely attenuated in seropositive adults and children but still caused some moderate respiratory tract disease in RSV-seronegative infants (12, 16, 18). These studies with humans indicated that the biologically derived *cp*RSV vaccine candidate possessed an attenuation phenotype, and this was subsequently confirmed in seronegative chimpanzees (8). *cp*RSV lacks an in vitro marker of attenuation since the virus is neither significantly cold adapted nor temperature sensitive (*ts*) in tissue culture (6, 8, 12) and therefore exhibits the host range phenotype; i.e., its replication is permissive in tissue culture but restricted in humans and chimpanzees. The chimpanzee is the only experimental animal that can be used to assess this attenuation phenotype.

To better understand the genetic basis for the attenuation of *cp*RSV, the sequence of the entire genome was determined and compared to that of wild-type (wt) RSV strain A2 (6). At the time this sequencing project was initiated, the exact parent of *cp*RSV, which had been passaged five times in human embryonic kidney (HEK) tissue culture, no longer existed, but a derivative which had been further passaged two times in HEK tissue culture (HEK-7) was available. Sequence analysis of *cp*RSV and RSV HEK-7 showed that *cp*RSV had accumulated five nucleotide substitutions, one in the N gene, two in the F gene, and two in the L gene, resulting in five predicted amino acid substitutions (6, 10) (Table 1). The recent development of a system for recovery of infectious virus from cDNA clones of RSV permits us to identify the genetic basis of attenuation of RSV vaccine candidates, as well as to develop new vaccine candidates (4). Therefore, the goal of the present study was to determine if the set of five *cp* mutations, which have been identified by sequence analysis, indeed confers the attenuation phenotype. This is of particular importance because *cp*RSV was the parent for a series of further-attenuated RSV strains (described below), and thus the *cp* mutation set is a common feature of current RSV vaccine candidates.

The five *cp* mutations were introduced together into a modified cDNA representing the RSV subgroup A genome by using our previously described methods (15). The antigenome cDNA that was the starting material for these experiments was cDNA D46, from which RSV was first recovered in 1995 (4). cDNA D46 differed in two ways from the natural A2 isolate from which the cDNA was prepared. First, four restriction enzyme cleavage sites (marker mutations) (Table 1) were created by a single nucleotide insertion in the NS2-N intergenic region (IGR) and five nucleotide substitutions, two in the N-gene noncoding region, two in the G-F IGR, and one in the F-M2 IGR (4). Second, a G-to-C (negative-sense) substitution at nucleotide 4 in the leader region (the 4C mutation)

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TABLE 1. Mutations introduced into full-length cDNA clones used to create recombinant RSV

Mutation	Gene	Nucleotide position[a]	Restriction marker (cleavage site position)	Sequence changes[b]	Amino acid position	Amino acid change
4C	Leader			A2 wt: ACGCGAAAAAATGCGTAC D46: ACGGGAAAAAATGCGTAC		Noncoding
Marker	NS2-N IGR		*Afl*II (1099)	A2 wt: AATTTAAAA.TTAAGGAG D46: AATTTAAAACTTAAGGAG		Noncoding
	N NCR		*Nco*I (1139)	A2 wt: CAAATACAAAG ATG GCT D46: CAAATACAACC ATG GCT		Noncoding
	G-F IGR		*Stu*I (5613)	A2 wt: ACAAAAAGCCATGACCAA D46: ACAAAAGGCCTTGACCAA		Noncoding
	F-M2 IGR		*Sph*I (7563)	A2 wt: CACAATTGAATGCCAGAT D46: CACAATTGCATGCCAGAT		Noncoding
Site	L		*Bsu*36I (9399)	A2 wt: TTA GGC TTA AGA TGC rA2sites: TTA GGC CTA AGG TGC		Silent
	L		*Sna*BI (11848)	A2 wt: GAA CCT ACA TAT CCT rA2sites: GAA CCT ACG TAT CCT		Silent
	L		*Pme*I (13342)	A2 wt: AAA CGT CTT AAT GTA rA2sites: AAA CGT TTA AAC GTA		Silent
	L		*Rsr*II (14083)	A2 wt: TTG CGT ACA GTA GTG rA2sites: TTG CGG ACC GTA GTG		Silent
	L		*Bst*EII (14318)	A2 wt: TTG TCT GTA ACA GTC rA2sites: TTG TCG GTA ACC GTC		Silent
	L		*Sna*BI (14477)	A2 wt: AAA ACT TAT GTA TGC rA2sites: AAA ACT TAC GTA TGC		Silent
HEK	F	5857	*Ssp*I (5850)	D46: AGT AAT ATC AAG *AAA HEK-7: AGT AAT ATC AAG GAA rA2cp: AGT AAT ATT AAG GAA	66	Lys→Glu
	F	5963	*Sca*I (5958)	D46: CAA AGC ACA *CAA GCA HEK-7: CAA AGC ACA CCA GCA rA2cp: CAA AGT ACT CCA GCA	101	Gln→Pro
cp	N	1939	*Cla*I (1936)	A2 wt: GCA AAA TCA *GTT AAA rA2cp: GCA AAA TCG ATT AAA	267	Val→Ile
	F	6314	*Nru*I (6313)	A2 wt: TCA AAT ATA *GAA ACT rA2cp: TCA AAT ATC GCG ACT	218	Glu→Ala
	F	7229	*Ase*I (7229)	A2 wt: TCC ACC *ACA AAT ATC rA2cp: TCC ACC ATT AAT ATC	523	Thr→Ile
	L	9454	Lose *Acc*I (9455)	A2 wt: GGA GAT *GT ATA CTA rA2cp: GGA GAT TAC ATA CTA	319	Cys→Tyr
	L	13566	*Hpa*I (13558)	A2 wt: CTA TTA ACT AAA *CAT rA2cp: CTG TTA ACT AAA TAC	1690	His→Tyr

[a] The nucleotide position shown is for nucleotide changes (indicated by an asterisk) leading to an amino acid substitution in the biologically derived wt RSV (HEK-7) and *cp*RSV. Numbering reflects the one nucleotide insertion in the NS2-N IGR of recombinant virus.

[b] Nucleotide changes (positive sense) are shown in boldface type. Restriction enzyme recognition sites are underlined.

(Table 1) was introduced since C at this position was previously found to be an up-regulator of RNA synthesis in an RSV minigenome system (13, 19a). This G-to-C substitution does not have an effect on the level of attenuation specified by a candidate RSV vaccine (11), an observation which is also confirmed here. Infectious virus recovered from this cDNA is designated rD46. cDNA D46 was then modified by the insertion of a set of six translationally silent restriction enzyme

TABLE 2. The set of five *cp* mutations attenuates RSV for the upper and lower respiratory tracts of chimpanzees

Virus used to infect animal[a]	Marker				Chimpanzee no.[b]	Mean virus titer (log_{10} PFU/ml)				Rhinorrhea score[d]		Days with cough
						Nasal wash sample		Tracheal lavage sample				
	4C	Site	HEK	*cp*		Peak titer	Daily titer[c]	Peak titer	Daily titer[c]	Peak	Mean	
rD46	X				1	4.9	3.7	3.4	3.2	3	1.1	4
	X				2	5.1	3.1	4.6	4.5	4	2.0	4
	X				3	4.4	3.9	4.0	2.5	3	1.4	5
	X				4	4.6	3.5	4.7	3.0	4	2.6	5
Mean ± SE						4.8 ± 0.16	3.6 ± 0.15	4.2 ± 0.30	3.1 ± 0.33	3.5	1.8	4.0
A2 wt[e]			X		5*	4.6	3.6	5.1	3.7	3	1.0	2
			X		6*	5.3	3.6	5.9	4.0	3	2.1	0
Mean ± SE						5.0 ± 0.35	3.6 ± 0.30	5.5 ± 0.40	3.8 ± 0.46	3.0	1.6	1.0
rA2sites	X	X			7	4.8	4.2	4.1	3.7	2	0.9	1
	X	X			8	5.4	3.8	5.5	4.1	3	1.4	3
	X	X			9	4.8	4.4	5.3	4.2	2	0.9	3
	X	X			10	4.9	3.7	3.8	2.9	3	2.0	3
Mean ± SE						5.0 ± 0.14	4.0 ± 0.18	4.7 ± 0.43	3.7 ± 0.33	2.5	1.3	2.5
rA2cp	X	X	X	X	11	4.3	2.1	4.5	3.3	2	1.1	0
	X	X	X	X	12	3.8	2.7	1.0	1.0	2	0.9	0
	X	X	X	X	13	4.6	3.2	3.7	3.2	0	0	0
	X	X	X	X	14	4.8	3.5	3.8	2.4	0	0	0
Mean ± SE						4.4 ± 0.22	2.9 ± 0.23	3.3 ± 0.77	2.7 ± 0.40	1.0	0.5	0.0
*cp*RSV[f]			X	X	15*	5.0	3.6	2.8	2.8	1	0.5	0
			X	X	16*	4.3	3.4	3.0	3.0	1	0.6	0
Mean ± SE						4.7 ± 0.35	3.5 ± 0.22	2.9 ± 0.10	2.9 ± 0.10	1.0	0.6	0.0

[a] Chimpanzees were inoculated by the intranasal and intratracheal routes with 10^4 PFU of the indicated virus in a 1-ml dose per site. Nasal wash samples were collected daily for 10 days, and tracheal lavage samples were collected on days 2, 5, 6, 8, and 10.

[b] *, historic control animal from the work of Crowe et al. (7).

[c] Mean daily titers were computed for each chimpanzee by adding together the virus titers from days with detectable levels of virus (≥0.7 PFU/ml) and dividing this sum by the total number of days with detectable virus titer.

[d] The amount of rhinorrhea was estimated daily and assigned a score (0 to 4) that indicated extent and severity. Scores indicate severe (4), moderate (3), mild (2), trace (1), or no (0) rhinorrhea. Mean rhinorrhea scores represent the sum of scores during the 8 days of peak virus shedding divided by 8.

[e] Biologically derived control virus (lot F-059) for which the presence of the HEK F-gene mutations was confirmed.

[f] Biologically derived *cp* virus (lot 3131).

cleavage sites (site mutations) (Table 1) which were created in the L gene to act as genetic markers for identification of recombinant virus and to aid in future construction of L-gene mutants. This cDNA, designated D53sites, was used to generate the recombinant RSV rA2sites (15). Finally, the set of five *cp* mutations was introduced together with two additional mutations (HEK mutations) (Table 1) required to bring the F-gene coding region of the recombinant virus into agreement with that of RSV HEK-7. These two changes in F were made because alignment of the sequences of cDNA D46 and the HEK-passaged strain A2 RSV revealed two predicted amino acid substitutions in the F protein (6). These two F-gene changes are the only significant differences between the two passage levels of the RSV A2 isolates: the RSV A2 derivative, HEK-7, which has remained in a freezer for most of the last three decades, and the further-passaged RSV A2 that is in use in our laboratory, which is related to HEK-7 but which has undergone numerous rounds of plaque purification and propagation predominantly in HEp-2 cells. It is thus remarkable that the two viruses have so few differences. With the introduction of the two coding changes in F, the cDNA-encoded virus is identical at the amino acid level to HEK-7. Each of the *cp* and the two HEK mutations introduced into cDNA were genetically marked by an accompanying translationally silent restriction enzyme cleavage site addition or deletion. Virus recovered from this cDNA was designated rA2cp and contained the original 4C and marker mutations found in rD46, the six L-gene restriction site markers, the two HEK F-gene mutations, and the set of five *cp* mutations. The rD46, rA2sites, and rA2cp mutants, like their biologically derived counterparts, produced plaques efficiently on monolayers of tissue culture cells at 40°C and therefore are non-*ts* viruses.

The attenuation phenotype of rA2cp was evaluated in RSV-seronegative chimpanzees as previously described (8) by comparing its levels of replication and pathogenicity with those of the rD46 and rA2sites wt recombinant viruses (Table 2). These findings, in turn, were compared to results from previous control studies with biologically derived *cp*RSV and wt RSV A2. Because of the severely limited number of available RSV-seronegative chimpanzees, test groups in this study were rela-

tively small and limited to four animals. Upper respiratory tract (nasal wash) and lower respiratory tract (tracheal lavage) samples were collected over a period of 10 days, and the chimpanzees were monitored daily for symptoms of rhinorrhea and cough. To compare the levels of virus replication of wt and attenuated viruses, we determined mean peak titers and mean daily titers. Whereas the peak titers compare the maximum levels of virus replication achieved in each animal, the mean daily titers (see Table 2, footnote *c*, for definition) estimate the total extent of replication. We generally consider differences in mean titer greater than 10-fold as significant. Rhinorrhea scores (see Table 2, footnote *d*, for definition) and cough symptoms were also compared. We have defined mean rhinorrhea scores greater that 1.0 as significant and consider any day with coughing as significant. The three wt viruses, rD46, rA2sites, and biologically derived A2 wt, were comparable in their levels of virus replication and in the extent of illness they caused in chimpanzees (Table 2). Likewise, the levels of virus replication and illness in chimpanzees infected with rA2cp and the biologically derived *cp*RSV were similar. This is so despite the 28 nucleotide differences between the two viruses, which represent the silent changes purposefully introduced into rA2cp. Specifically, the mean peak or daily virus titers in either the upper or lower respiratory tract did not appear to be significantly different between rA2cp and biologically derived *cp*RSV. Importantly, each of the two *cp* viruses replicated less well and induced fewer symptoms than the wt viruses.

The rA2cp virus was directly compared with its most closely related recombinant wt virus, rA2sites. In the lower respiratory tract, the rA2cp mutant virus exhibited a 25-fold decrease in peak virus titer as well as a 10-fold decrease in mean daily virus titer compared to the titers for the rA2sites virus. In the upper respiratory tract, there were also modest 4- and 10-fold decreases in the peak virus titer and mean daily titer, respectively. In comparison with chimpanzees receiving wt rA2sites, those inoculated with rA2cp showed a marked decrease in rhinorrhea and cough. These findings with rA2cp demonstrate that the set of five *cp* mutations indeed specifies the host range, attenuation phenotype. Unfortunately, due to the limited availability of RSV-seronegative chimpanzees, it is not feasible at this time to examine the contribution of individual *cp* mutations to overall virus attenuation and prevention of illness associated with infection.

The design of these studies also allows for an evaluation of the effects of the other sets of introduced mutations on virus replication or illness. The original recombinant virus, rD46, was comparable in replication and virulence to the biologically derived A2 wt virus (Table 2, compare results for chimpanzees 1 to 4 with chimpanzees 5 and 6). This shows that this recombinant virus, on which all subsequent engineered viruses will be based, indeed is wt with respect to replication and virulence in a fully permissive experimental animal. Thus, it does not contain any incidental deleterious changes, and furthermore the four marker mutations and the 4C mutation do not significantly alter its properties or that of its rA2cp derivative. In addition, since the mean virus titers of rA2sites and rD46 are not significantly different, the six translationally silent L-gene site mutations do not appear to affect virus replication in this permissive host (Table 2, compare results for chimpanzees 1 to 4 with chimpanzees 7 to 10). Similarly, the HEK F-gene mutations do not appear to modify virulence (Table 2, compare results for chimpanzees 5 and 6 with chimpanzees 1 to 4 and 7 to 10). However, since the HEK-7 RSV was the genetic background for biologically derived *cp*RSV, it is possible that the *cp* mutations may interact with these HEK F-gene mutations. Since this is not directly tested here, the HEK F-gene mutations will be included with the *cp* mutations in future constructs.

Our approach to the development of a live attenuated vaccine virus is to sequentially introduce both *ts* and non-*ts* attenuating mutations into the genome of wt RSV until a proper balance between attenuation and immunogenicity has been achieved. The rationale for this design is based on the observation that several successful, live attenuated vaccines and vaccine candidates, including those for polioviruses, orthomyxoviruses, and paramyxoviruses, have *ts* mutations accompanied by non-*ts* mutations, both of which contribute to their attenuation (1, 8, 14, 17, 22, 23, 25). Because several live attenuated candidate vaccines that contain only *ts* mutations contributing to their attenuation readily undergo loss of their temperature sensitivity in animals or humans (7, 19, 22, 24), it was considered prudent to stabilize the *ts* and attenuation phenotypes of candidate RSV vaccines by combining both *ts* and non-*ts* attenuating mutations. Since the set of five *cp* mutations indeed specifies the attenuation phenotype, it represents our first non-*ts* attenuating genetic element for RSV. A second non-*ts* attenuating mutation, the deletion of the small hydrophobic (SH) protein of RSV, has also recently been identified (2). We are in the process of identifying the genetic basis of the attenuation and temperature sensitivity of a panel of *ts* viruses, such as *cpts*-248/404, *cpts*-530/1009, and *cpts*-530/1030, which were derived from *cp*RSV and show a range of phenotypes with regard to temperature sensitivity and attenuation (7, 8, 9, 11, 15). The *ts* mutations identified in these studies and the non-*ts* SH deletion mutation are currently being added individually and in combination to rA2cp to assess their contribution to attenuation and to create a new generation of novel live attenuated virus vaccine candidates. In this way, we believe that it will be possible to derive a satisfactorily attenuated and immunogenic RSV vaccine candidate in the near future.

Nucleotide sequence accession number. The nucleotide sequence of rA2cp has been submitted to the GenBank nucleotide sequence database and assigned accession no. AF035006.

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Evaluation of a Live, Cold-Passaged, Temperature-Sensitive, Respiratory Syncytial Virus Vaccine Candidate in Infancy

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A live-attenuated, intranasal respiratory syncytial virus (RSV) candidate vaccine, *cpts*-248/404, was tested in phase 1 trials in 114 children, including 37 1–2-month-old infants—a target age for RSV vaccines. The *cpts*-248/404 vaccine was infectious at 104 and 105 plaque-forming units in RSV-naive children and was broadly immunogenic in children >6 months old. Serum and nasal antibody responses in 1–2 month olds were restricted to IgA, had a dominant response to RSV G protein, and had no increase in neutralizing activity. Nevertheless, there was restricted virus shedding on challenge with a second vaccine dose and preliminary evidence for protection from symptomatic disease on natural reexposure. The *cpts*-248/404 vaccine candidate did not cause fever or lower respiratory tract illness. In the youngest infants, however, *cpts*-248/404 was unacceptable because of upper respiratory tract congestion associated with peak virus recovery. A live attenuated RSV vaccine for the youngest infant will use *cpts*-248/404 modified by additional attenuating mutations.

Respiratory syncytial virus (RSV) is the leading cause of severe viral respiratory disease in infants and children [1]. It is also an important cause of severe respiratory disease in the elderly [2], immunocompromised patients of all ages [3, 4], and infants with underlying cardiopulmonary disease [5]. It is considered a major infectious trigger for reactive airway disease [6]. RSV infections are estimated to account for ~90,000 pediatric hospitalizations and 4500 deaths yearly in the United States [7]. RSV causes a yearly epidemic during the winter months, with a high penetrance in the first years of life. Of the 2 serologically distinct subgroups, RSV A and B, RSV A viruses appear to be slightly more virulent and are more commonly isolated [8]. To be effective, a vaccine must protect against RSV-associated lower respiratory tract disease in very young infants, because the peak age of hospitalization is in the second and third months of life [9].

To introduce an RSV vaccine into the pediatric immunization schedule, the following properties of the vaccine must be assessed: (1) its safety in infancy, (2) the effect of maternal antibody on its infectivity, (3) the effect of immunological immaturity or transplacental maternal antibody on its immunogenicity [10, 11], and (4) its efficacy against natural RSV infection. By analogy with vaccines given in infancy against other pathogens, multiple doses are expected to be required.

Live, attenuated, intranasally administered RSV vaccines have been under development since the late 1960s. At that time, the parent strain of the lineage under current investigation (cold-passaged [*cp*] RSV) was evaluated in seronegative children as young as 2 years old [12]. In seronegative children, *cp* RSV caused mild respiratory illness that was temporally associated with virus shedding [13].

In parallel, there was an effort to develop a formalin-inactivated vaccine. This inactivated vaccine failed to protect vaccine recipients and led to enhanced illness on natural exposure to virus [14]. That enhanced illness has profoundly influenced the

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Informed consent was obtained from parents or guardians of volunteers, and the human experimentation guidelines of the US Department of Health and Human Services and of the relevant institutions were followed in the conduct of the clinical research.

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development of an RSV vaccine by delaying the evaluation of other inactivated or subunit vaccines that might follow similar antigen processing and presentation pathways and have attendant safety concerns [15]. Consequently, live vaccines that mimic natural infection have been pursued as the safest strategy for immunizing young children. Wild-type (wt) RSV infection does not exhibit enhanced disease during reinfection. Intranasal infection should induce mucosal immunity, which contributes to protection against RSV [16, 17] and should lead to a balance between Th1 and Th2 immunological responses without the risk of enhanced illness [18].

Recently, a series of further attenuated candidate vaccines [19–21] were derived by chemical mutagenesis of *cp* RSV. *cp* RSV was subjected to 2 rounds of chemical mutagenesis, and temperature-sensitive *(ts)* mutant derivatives were generated. The non–*ts*-attenuating mutations of the *cp* RSV parent virus were anticipated to work in concert with the subsequently introduced *ts* mutations, to yield further attenuated, genetically stable viruses. The *ts* phenotype of the *cpts* vaccine is more stable than that of a prototype *ts* virus, RSV *ts*-1, which also was evaluated in children [22, 23]. The mutations in the *cpts* vaccines are distinct from mutations in the overly attenuated RSV *ts*-2 that block replication in the human respiratory tract [24]. The first 2 vaccines evaluated in the *cpts* lineage, *cpts*-248/955 and *cpts*-530/1009, were either insufficiently attenuated or were transmitted among seronegative children 6–36 months old [25].

The present candidate, *cpts*-248/404, is among the most attenuated of the current series of *cpts* vaccines on the basis of in vitro and in vivo analysis. Because its shut-off temperature for plaque formation is between 35°C and 36°C, it is unlikely to replicate efficiently at human core body temperature, 37°C. The *cpts*-248/404 vaccine is highly restricted in its replication in chimpanzees. Only $10^{1.3}$ pfu/mL was recovered from the upper respiratory tract (levels >1000-fold lower than that of wt RSV), and virus was not recovered from the lungs, despite the fact that infection was initiated by direct intratracheal instillation. When passive RSV antibodies were given before immunization of 2 chimpanzees to mimic transplacental maternal antibodies, an inapparent infection with *cpts*-248/404 occurred without documented virus shedding and a limited antibody response that, nevertheless, protected these animals against challenge with wt RSV 28 days later. There was an enhanced neutralizing antibody response after challenge with wt RSV [19].

In the present study, *cpts*-248/404 underwent testing in progressively younger children, which resulted in the first administration of a live, attenuated RSV vaccine to the target age group for vaccination of infants—namely, those <2 months old. In the youngest age group, *cpts*-248/404 vaccine caused mild-to-moderate upper respiratory congestion, precluding it from being a candidate for efficacy trials in early infancy. Nevertheless, valuable information was derived from this study, including the quantity of vaccine virus required to infect a 1–2-month old infant, the level of vaccine virus replication in the presence of maternal antibodies, immunogenicity in the presence of maternal antibodies, ability of the first dose of vaccine to restrict replication of a second dose, transmissibility, phenotypic stability of the *cpts*-248/404 vaccine, the level of attenuation of RSV vaccines that is needed for the very young infant, and preliminary evidence of protection from symptomatic illness during natural reexposure to wt RSV.

Materials and Methods

Viruses. Isolation and characterization of *cpts*-248/404 (shut-off temperature for plaque formation of 35°C–36°C) has been described elsewhere [19, 20]. A viral suspension for clinical trials was produced in Vero cells and found to be free of adventious agents by Dr. Louis Potash (lot RSV A-25; Nova Vax, Bethesda, MD). The titer of *cpts*-248/404 (lot RSV A25) prepared for clinical evaluation was $10^{5.3}$ pfu/mL. When necessary to achieve the planned titer for inoculation, this virus suspension was diluted in L-15 medium (BioWhittaker, Walkersville, MD). Nineteen vaccine and 11 placebo recipients in the 6–24-month-old group received *cpts*-248/404 vaccine (lot LRSV-404-301) prepared by Wyeth-Lederle Vaccines and Pediatrics (Pearl River, NY) that had a titer of 10^8 pfu/mL. To achieve the planned titer for inoculation, this virus suspension was diluted in phosphate buffered saline with sucrose-phosphate-glutamate. The results with both vaccine lots are similar, and the results are combined.

Clinical studies in children. Because the parental strains from which the further attenuated *cpts*-248/404 was derived had very limited infectivity in adults [12, 25], adult studies were not performed, and initial studies were done in seropositive children 15–59 months old. Children were enrolled in this randomized, double-blind, placebo-controlled phase I study at 3 study sites, with a 2:1 ratio of vaccine to placebo recipients. Children were eligible to participate if they were healthy and if all other family members and day care contacts were ≥6 months old. Before enrollment, children were screened for the presence of RSV serum-neutralizing antibodies by a complement-enhanced, 60% plaque reduction neutralization test [26]. Those with titers >1:40 were considered RSV seropositive. Each study subject received 0.5 mL of vaccine or placebo as intranasal drops. Children were examined daily for 10 days after vaccine administration. Each study group of 4–6 children was unblinded after clinical observations were completed, so that continuous monitoring of safety could be maintained among sites.

Because of the high level of attenuation of a dose of 10^5 pfu in 17 seropositive children, studies were then performed in 74 seronegative children 6–24 months old at a dose of 10^5 or 10^4 pfu. The first 31 children immunized with 10^5 pfu of RSV *cpts*-248/404 vaccine were examined on days 0–10 and day 14. Subsequently, the remaining children immunized with 10^5 pfu and all children immunized with 10^4 pfu were examined and had samples taken on days 0, 4 or 5, 7 or 8, and 10 or 11. Using the latter schedule, studies then were done in infants 3–5 months old at a dose of 10^5 pfu and then in 1–2-month old infants at a dose of 10^5 pfu or 10^4 pfu. Although some infants had maternally derived antibodies in their serum, these study subjects had not been previously infected with RSV, because they were born after the previous annual RSV epidemic. One month after the first dose of *cpts*-248/404, all avail-

able 1–2-month-old vaccine and placebo recipients were given a second dose of the vaccine or placebo to which they were originally randomized.

Children were observed for 1–2 h on each study day in an outpatient setting. Febrile illness was defined as a rectal temperature ≥38.1°C. Respiratory illness was categorized as upper respiratory tract infection (URI), defined as rhinorrhea or pharyngitis of ≥2 consecutive days duration, or lower respiratory tract infection (LRI), defined as persistent rhonchi, rales, or wheezing. Cough for ≥2 consecutive days was recorded without assignment as to the site of involvement of the respiratory tract [23].

Isolation, quantitation, and identification of virus. Nasal wash specimens for virus isolation were obtained on each day of observation from all subjects and from those with illness reported in the 3 weeks after immunization, as described elsewhere [25]. Fresh or snap-frozen samples were inoculated into 2 sets of tissue culture tubes that contained either Vero or HEp-2 tissue culture cell monolayers and were incubated at 32°C. Viral isolates from these cultures were identified as RSV by use of an indirect immunofluorescence assay (IFA; Bartels Microscan; Baxter Healthcare, Bellevue, WA). RSV in fresh or snap-frozen specimens was titered by plaque assay on HEp-2 cell monolayer cultures maintained under a semisolid overlay at 32°C, as described elsewhere [24], and results were expressed as $\log_{10}$ pfu/mL. For purposes of calculation, samples in which virus was not detected were assigned a titer of $10^{0.6}$ pfu/mL.

Phenotypic characterization of viral isolates. The level of temperature sensitivity of virus present in snap-frozen nasal wash specimens was determined by plaque titration in HEp-2 cell monolayers at 32°C, 36°C, 37°C, 38°C, and 39°C, as described elsewhere [25]. Virus isolates from nasal wash samples that showed a significantly altered *ts* phenotype (an increase in shut-off temperature for plaque formation ≥2°C) were prepared for analysis by either 2 passages on HEp-2 monolayers or by 1 round of plaque purification on HEp-2 cell monolayers. The level of attenuation of isolates with altered *ts* phenotype was assessed by examining their level of replication in BALB/c mice, as described elsewhere [27].

Genetic characterization of viral isolates. Viral isolates that showed an altered *ts* phenotype were further characterized by sequence analysis. Reverse transcription–polymerase chain reaction (RT-PCR) amplification of viral RNA was performed for regions of *cpts*-248/404 known to contain determinants of the *ts* and attenuation phenotypes, as described elsewhere [25]. The nucleotide sequence of mutations specific to *cp* RSV and *cpts*-248/404 was determined by use of Sequenase 2.0 (USB, Cleveland, OH), as described by Whitehead et al. [27].

Immunological assays. Sera and nasal wash specimens were obtained before and either 4 or 8 weeks after initial immunization for measurement of RSV-specific antibodies. A third serum and nasal wash specimen was obtained 1 month after the second dose from study subjects who received either vaccine or placebo at 1–2 months old. For comparison, additional sera and nasal wash samples were available from 18 children 1–2 months old who were not enrolled in these trials but were hospitalized for illness caused by wt RSV infection. Sera were tested for antibodies to RSV by a plaque neutralization assay [26] and for IgG and IgA antibodies to RSV fusion (F) and attachment (G) proteins by an end-point titration in a modification of an ELISA described elsewhere [25]. In brief, Nunc polysorb plates were coated with 20 ng/well of either purified F or G protein in carbonate buffer and were blocked with 0.5% gelatin in phosphate buffered saline with 0.05% Tween (PBST). Sera were diluted in PBST with 0.5% gelatin and 2% fetal calf serum on antigen-coated and noncoated wells. After 1 h of incubation, the plates were washed and were incubated with goat anti–human IgG or IgA alkaline phosphate conjugate (Jackson ImmunoResearch, West Grove, PA) for 1 h. Color development used 1 mg/mL of D-nitrophenylphosphate (Sigma, St. Louis) in diethanolamine buffer. Optical densities (OD) were read at 405 nm wavelength, the OD was subtracted for the corresponding blank well, and the end-point dilution at 0.2 OD was calculated. The results were expressed as $\log_2$ of end point, with a positive response defined as a ≥4-fold increase in antibody titer.

Nasal wash samples were tested for the presence of IgA antibodies to purified RSV F and G proteins by a kinetic ELISA (kELISA) originally developed for influenza [28]. The increase in absorbance in milliOD/min of each nasal wash IgA value was expressed as a dilution of a standard serum curve run in the assay that gave the same increase in absorbance. kELISA values <5 milliOD/min were defined as negative. For pre- and postsamples with RSV-specific antibody, the RSV-specific results were adjusted for their total IgA concentration, as measured by a radial immunodiffusion assay using secretory IgA standards (Binding Site, San Diego, CA). Replicate experiments determined that a ≥4-fold difference in the standardized, adjusted result was at the 95% confidence interval (CI) for the test. Therefore, pre- to postimmunization changes of this magnitude or paired samples that went from negative to positive were considered to demonstrate a mucosal antibody response.

Surveillance. By use of methods described elsewhere [25], RSV vaccine recipients, placebo recipients, and age-matched control subjects were monitored during the subsequent RSV season for the occurrence of wt RSV–associated illness, to determine whether immunization with live attenuated vaccine prevented RSV disease or modified the clinical response to subsequent infection with RSV. Parents were contacted on a weekly basis throughout the time when wt RSV was identified in the 3 groups, in which trials were conducted. If a child developed a respiratory illness that met one of the definitions used during the initial vaccine evaluation, the child had a clinical assessment and viral culture. Before and after the RSV epidemic season, children had serum drawn to measure the incidence of RSV infection in the study population, as judged by increases in serum neutralizing antibody titer. The surveillance was not blinded; participant families and investigators knew the child's vaccine status.

Data analysis. Infection with RSV *cpts*-248/404 vaccine was defined as the isolation of *cpts* RSV, a ≥4-fold increase in serum RSV neutralizing antibody titer, and/or a ≥4-fold increase in ≥2 of the ELISA-based assays. In infants with residual maternal antibodies at the time of immunization, an increase was calculated as being 4-fold above the anticipated 28 day half-life ($t_{1/2}$) of passive antibodies. All comparisons of antibody titer by age were made after the first dose of vaccine. Titers were expressed as reciprocal mean $\log_2$. The Mann-Whitney *U* test (2-tailed) was used to compare the mean titers among the groups. Comparison of tabulated data (e.g., comparison of frequency of illness among vaccine and placebo recipients and comparison of immunological responses) was made using 2-tailed Fisher's exact tests. The κ statistics were

computed to measure concordance of antibody responses. The associations among antibody response, age, and prevaccine antibody were estimated using logistic regression analysis.

Results

Response of seropositive 15–59-month-old children to cpts-*248/404.* In this age group, the frequency of illness was comparable between the 11 vaccine and 6 placebo recipients (table 1). Virus was not recovered from seropositive vaccine recipients given 10^5 pfu, and the only antibody response was seen in a single study subject who had an increase in mucosal IgA antibody to the RSV G protein (table 2). Therefore, RSV *cpts*-248/404 is the most restricted in the *cpts* series evaluated, to date, in regard to infectivity for seropositive children.

Response of 6–24-month-old seronegative infants and children to RSV cpts-*248/404.* The *cpts*-248/404 vaccine was highly infectious and immunogenic in 6–24-month-old seronegative vaccine recipients. After a single administration of either 10^4 or 10^5 pfu, ~90% of vaccine recipients exhibited evidence of infection (tables 1 and 2). There was no difference in the frequency of illness seen in the vaccine and placebo recipients, although the ability to detect differences was limited by the high frequency of minor illness seen in children of this age group over a 10-day period of close observation. Notably, 1 vaccine recipient had mild wheezing associated with shedding of *cpts* RSV, and 2 vaccine recipients had rhonchi (1 with evidence of RSV infection). One placebo recipient developed mild wheezing temporally associated with an adenovirus infection.

Vaccine recipients given the 10^5 dose of vaccine shed virus with high frequency (79%) and at a moderately high level. The mean peak titer was $10^{4.2}$ $\log_{10}$ pfu/mL of nasal wash. The lower mean peak titer of vaccine virus ($10^{2.4}$ $\log_{10}$ pfu/mL) shed by recipients of the 10^4 dose may be methodological, because the samples were collected by nasal swabs rather than by nasal washes. Thirty-four of the 45 6–24-month-old vaccine recipients tested who had been given 10^4 or 10^5 pfu developed a serum-neutralizing antibody response, with an increase in mean neutralizing titer to ~1:300. The dose of vaccine did not influence the frequency or magnitude of the antibody response (table 2). Serum IgG ELISA responses to F and G proteins were concordant with neutralizing antibody responses (κ values of .76 and .74, respectively).

Response of 3–5-month-old infants to RSV cpts-*248/404.* In the next study, 16 infants (10 vaccine and 6 placebo recipients) 3–5 months old were given 10^5 pfu of *cpts*-248/404. One vaccine recipient developed a fever of 38.2°C, but LRI was not seen. There was a suggestion that URI was more frequent in vaccine recipients (7 of 10) versus placebo recipients (2 of 6; $P = .30$; table 1). Vaccine virus was recovered from 3–5-month old vaccine recipients, including 5 with residual maternal neutralizing antibodies, at levels comparable with older RSV-naive infants (figures 1 and 2). This finding suggests that maternally acquired serum antibody did not restrict nasal replication of the attenuated *cpts* vaccine, which is an observation confirmed in the youngest age group (figure 2). Systemic and mucosal immune responses were lower in frequency and in magnitude than those observed for vaccine recipients >6 months old; however, the

Table 1. Clinical and virological responses of infants and children to respiratory syncytial virus (RSV) *cpts*-248/404 or placebo.

			Virus isolation (nasal wash)		Percentage with indicated illness				
Study subjects (age), dose in pfu	Virus given	*n*	Shedding, %	Mean peak titer, $\log_{10}$ pfu/mL[a]	Fever	URI	LRI	Cough	Otitis media
Seropositive children (15–59 months)									
10^5	*cpts*-248/404	11	0	0.6[b]	18	36	0	0	9
	Placebo	6	0	0.6[b]	33	33	17	17	0
Seronegative children (6–24 months)									
10^5	*cpts*-248/404	38	79	4.2	29	68	8	24	11
	Placebo	20	0	0.6[b]	40	70	5	30	30
10^4	*cpts*-248/404	11	55	2.4	18	100	0	36	9
	Placebo	5	0	0.6[b]	20	60	0	20	20
Infants (3–5 months)									
10^5	*cpts*-248/404	10	70	4.2	10	70	0	10	0
	Placebo	6	0	0.6[b]	0	33	0	0	17
Infants (1–2 months)									
Dose 1									
10^5	*cpts*-248/404	17	76	4.0	0	65	0	18	6
	Placebo	8	0	0.6[b]	0	0	0	0	0
10^4	*cpts*-248/404	7	100	4.9	14	86	0	43	0
	Placebo	3	0	0.6[b]	0	33	0	33	0
Dose 2									
10^5	*cpts*-248/404	15	27	3.8	7	40	0	7	0
	Placebo	7	0	0.6[b]	14	0	0	0	0
10^4	*cpts*-248-404	7	0	0.6[b]	0	33	0	0	0
	Placebo	3	0	0.6[b]	0	33	0	0	0

NOTE. LRI, lower respiratory tract infection; URI, upper respiratory tract infection.

[a] Calculated for infected volunteers only.

[b] Lowest limit of detection of the assay was 0.7 $\log_{10}$ pfu/mL. For samples without detectable plaques, a titer of 0.6 was assigned.

Table 2. Immunological responses of infants and children to respiratory syncytial virus (RSV) *cpts*-248/404 or placebo.

Subjects (age), dose in pfu	Virus given	*n*	Percentage infected[a]	RSV serum neutralizing antibody response: Titer, reciprocal mean $\log_2$ Before	After	≥4-Fold increase, %	Antibody response to indicated RSV protein: Serum IgG ELISA, reciprocal mean $\log_2$: F protein Before	After	≥4-Fold increase, %	G protein Before	After	≥4-Fold increase, %	Serum IgA ELISA, reciprocal mean $\log_2$: F protein Before	After	≥4-Fold increase, %	G protein Before	After	≥4-Fold increase, %	Nasal wash IgA ELISA: F protein ≥4-Fold increase, %	G protein ≥4-Fold increase, %
Seropositive children (15–59 months)																				
10^5	*cpts*-248/404	11	0	9.3	8.8	0	14.1	13.9	0	12.0	11.5	0	ND	ND	ND	ND	ND	ND	0	9
	Placebo	6	0	8.0	8.1	0	13.4	13.3	0	9.3	9.2	0	ND	ND	ND	ND	ND	ND	0	0
Seronegative children (6–24 months)																				
10^5	*cpts*-248/404	38	87	3.3	7.3	68	8.1	12.5	71	6.5	11.6	77	3.0	6.4	71	2.7	6.4	74	78	72
	Placebo	20	10	3.5	3.6	0	8.5	8.2	12.5	6.8	6.6	12.5	3.6	3.2	6.3	2.7	2.8	0	0	11
10^4	*cpts*-248/404	11	91	4.6	8.5	91	8.7	13.0	91	6.5	11.6	91	3.5	5.9	55	2.0	5.8	82	33	55
	Placebo	5	0	4.6	4.6	0	9.3	8.3	0	7.2	6.2	0	3.2	3.8	20	2.0	2.0	0	25	20
Infants (3–5 months)																				
10^5	*cpts*-248/404	10	90	5.0	5.8	44	9.8	10.2	78	9.0	8.9	56	2.7	3.5	22	2.3	4.2	56	22	56
	Placebo	6	0	4.8	3.8	0	10.2	8.2	17	10.2	8.2	17	3.1	3.5	17	2.5	3.0	17	0	17
Infants (1–2 months)																				
Dose 1																				
10^5	*cpts*-248/404	17	76	5.9	4.4	0	14.7	13.2	0	12.2	11.6	7	1.9	3.0	33	21	5.5	73	11	27
	Placebo	8	0	6.6	4.7	0	14.2	12.9	0	11.9	11.2	0	2.2	2.2	0	2.1	2.1	0	0	14
10^4	*cpts*-248/404	7	100	5.8	3.7	0	12.5	12.1	0	11.3	10.9	0	2.3	4.5	57	2.4	7.3	100	50	100
	Placebo	3	0	6.0	5.1	0	12.2	12.3	33	10.8	10.1	0	2.3	2.3	0	2.5	2.5	0	0	0
Dose 2[b]																				
10^5	*cpts*-248/404	15	33	4.6	4.7	13	13.2	12.9	20	11.6	11.6	20	3.0	4.0	33	5.5	6.3	47	18	45
	Placebo	7	0	4.7	3.8	0	12.9	11.7	0	11.2	10.2	0	2.0	2.2	0	1.9	2.1	0	0	0
10^4	*cpts*-248/404	7	14	3.7	4.4	29	12.1	11.4	0	10.9	11.1	29	7.3	7.3	29	7.3	7.3	29	17	17
	Placebo	3	0	5.1	4.3	0	12.3	12.5	33	10.1	10.9	33	2.3	2.4	0	2.5	2.4	0	0	0

NOTE. Data indicate no. of volunteers enrolled in the specified treatment group. Serology results and the calculated percentages with ≥4-fold increases in the associated columns indicate data obtained from testing the available paired samples, which, in all cases, represented ≥90% of the volunteers in that group. Paired nasal wash specimens were available in ≥75% of each group. After, after vaccination; Before, before vaccination; ND, not determined.

[a] Percentage infected was calculated using evidence by either serology or virus shedding, as described in Materials and Methods.

[b] Percentage of increases for these specimens are calculated to reflect increases specifically occuring with dose 2.

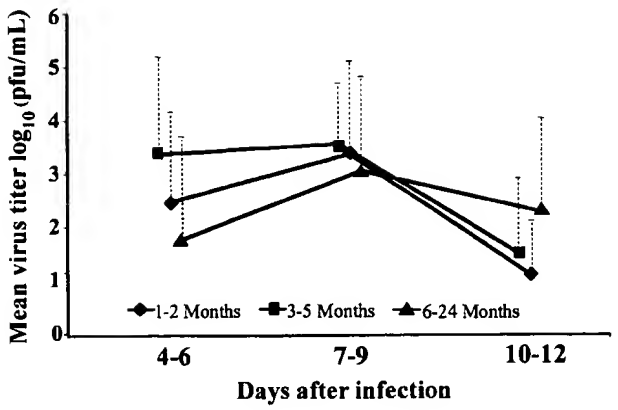

Figure 1. Recovery of respiratory syncytial virus (RSV) *cpts*-248/404 from the upper respiratory tract of RSV-naive infants and young children on selected days after vaccine administration. Mean titer of virus shed by children 1–3 months old (♦), 3–5 months old (■), and 6–24 months old (▲). SD is shown by upward deflection lines.

small number of 3–5-month-old children did not allow for statistical comparisons.

Response of 1–2-month-old infants to initial immunization. In a 2-dose regimen, 10^5 and 10^4 doses of vaccine were given to infants 1–2 months old, which is the target age group for RSV vaccination. Twenty-five children were studied; 17 received 10^5 pfu of vaccine, 7 received 10^4, and 11 received placebo (table 1). Seventeen of the vaccine recipients developed a clinical syndrome characterized by nasal congestion that occurred most typically between days 8 and 12. There were no signs of LRI on repeated examinations, although 6 mothers of vaccine recipients reported that their infants had a mild cough. Symptoms of congestion were linked temporally with the peak of virus shedding. Fifteen of 18 infants who shed $>10^3$ pfu of RSV per milliliter of nasal wash experienced congestion, fussiness while trying to sleep, and mild difficulty with feeding, which lasted ~24 h. Virus shedding was not accompanied by the profuse rhinorrhea typically seen with wt RSV infection, nor did any of the infants have LRI, otitis media, or fever. One of 11 placebo recipients and 1 of 4 vaccine recipients who did not shed virus had mild congestion on days 2 and 3 after receiving vaccine B, a significant difference in the rate of illness, compared with that of vaccine recipients from whom *cpts*-248/404 was recovered ($P = .0002$). The same pattern and frequency of congestion was seen in 10^5 and 10^4 vaccine recipients.

Thirteen of 17 vaccine recipients shed virus after the first 10^5 dose. All 7 vaccine recipients receiving 10^4 pfu shed virus in an amount equivalent to that observed for vaccine recipients receiving 10^5 pfu. In figure 1, the magnitude of virus shedding is compared among RSV-naive infants 6–24 months, 3–5 months, and 1–2 months old on days 4–6, 7–9, and 10–12 (constant days of sampling throughout the study). Peak virus shedding was similar for all ages. The lack of a correlation of the peak virus shedding in each infant <6 months old with his/her age (figure 2*A*) or level of maternal derived antibody (figure 2*B*) demonstrates that virus replication in the nasopharynx was independent of these variables.

Even when the expected decay in maternal antibody was considered in the calculations, 1–2-month-old vaccine recipients receiving either dose of vaccine rarely developed an increase in serum neutralizing antibody or an IgG ELISA response to F or G protein (table 2). In 18 infants of the same age hospitalized with wt RSV, infection serum neutralizing responses were also seen infrequently; only 30% had a ≥4-fold increase. In contrast, 29 of 35 6–24-month-old vaccine recipients who shed virus developed a neutralizing antibody response, which is significant when compared with the 1–2-month-old vaccines ($P < .001$).

In contrast to their failure to develop a serum neutralizing and IgG-based ELISA antibody responses, the 1–2-month-old vaccine recipients frequently developed a serum IgA response to RSV G and F glycoproteins (table 2 and figure 3). The frequency and magnitude of the IgA antibody response to the G glycoprotein of the 1–2-month-old vaccine recipients did not differ from that of 6–24-month-old vaccine recipients. In contrast, the frequency of the IgA response to the F glycoprotein, combining the results of 10^4 and 10^5 doses by the 1–2-month-old vaccine recipients (41%), was less than that of the 6–24-month-old vaccine recipients (67%) and also less than that to the G glycoprotein (82%) in the 1–2-month-old vaccine recipients. Therefore, these findings indicate that 1–2-month-old vac-

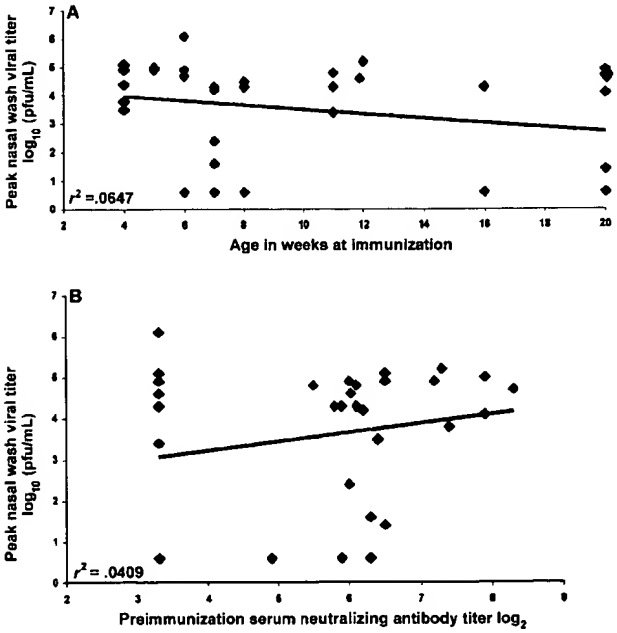

Figure 2. Peak recovery of respiratory syncytial virus (RSV) *cpts*-248/404 from the upper respiratory tract is independent of age *(A)* or level of maternally acquired serum antibody *(B)*. ♦, Individual volunteers.

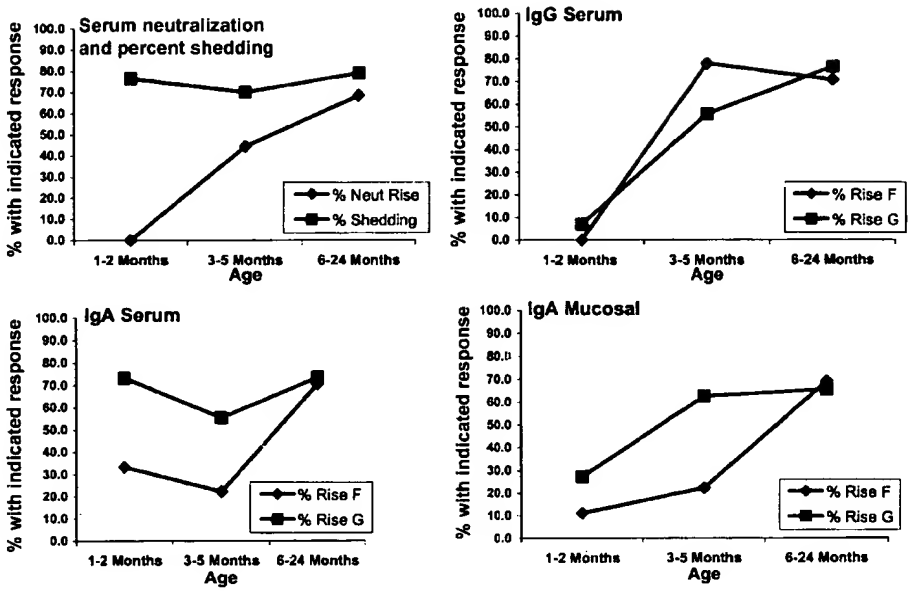

Figure 3. Maturation of the systemic and mucosal immune responses to respiratory syncytial virus *cpts*-248/404 vaccine with age. Percentage of children with a systemic or mucosal antibody response to the indicated immunoglobulin isotype is shown graphically by age. F, fusion protein; G, attachment protein.

cine recipients preferentially respond to the RSV G protein with an IgA response and that this response was similar to that of older vaccine recipients and was not influenced by titer of maternally derived antibodies.

Mucosal IgA responses were also seen in the 1–2-month-old infants (table 2 and figure 3). The mucosal response to G protein was also more frequent than that to F protein. Study of 18 infants of the same age hospitalized with wt RSV detected IgA mucosal responses in 61% to the F protein and 50% to the G protein, which are frequencies slightly higher than that observed for 1–2-month-old vaccine recipients. Neutralizing activity to RSV could be not detected in 10 available postvaccination nasal washes from 1–2-month-old vaccine recipients when tested at a dilution of 1:4.

Response of 1–2-month-old infants to second dose of vaccine. The relatively mild nature of the symptoms associated with the first dose allowed us to give the planned second dose of vaccine or placebo 4–6 weeks later to 22 available vaccine recipients (15 who received 10^5 and 7 who received the 10^4 dose) and to 10 placebo recipients (table 1). Virus was recovered from only 2 of 19 vaccine recipients who had shed virus after the first dose. Two of 3 who had not shed virus after the first dose of vaccine were infected, leaving 1 child who did not shed virus after either dose. Nasal congestion was observed in each of the vaccine recipients who shed RSV, but only in 2 of 18 vaccine recipients from whom virus was not recovered and in 1 of 10 placebo recipients ($P = .002$). The inverse relationship between peak virus shedding with the first and second dose of vaccine is shown in figure 4. Infection with the first dose provided a high level of resistance to replication of the second dose. There was an absolute correlation of protection from reinfection with detection of serum IgA antibody to the G protein ($P < .0001$), although, whenever an immune response to the first dose was documented by any assay in either serum or nasal wash, reinfection did not occur (table 3). Antibody increases after the second dose of vaccine were largely restricted to those vaccine recipients not infected with the first dose (table 2).

Factors influencing the immune responses of infants <24 months old to cpts-*248/404 vaccine.* Multivariate analysis of

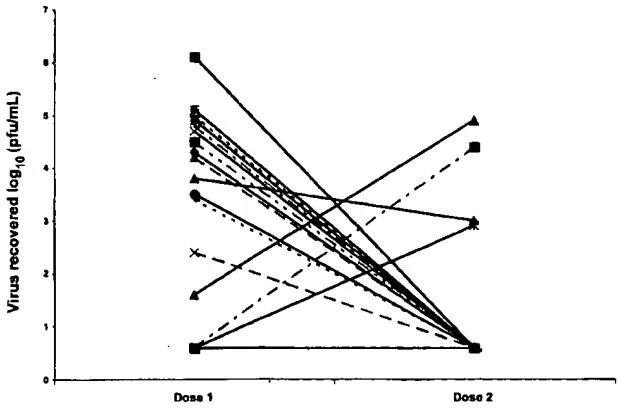

Figure 4. Influence of peak virus shedding on virus recovery, with the initial dose of respiratory syncytial virus *cpts*-248/404 given at 1–2 months old and a second dose of vaccine given 1 month later.

Table 3. Correlates of protection against a second dose of respiratory syncytial virus (RSV) *cpts*-248/404 vaccine when the initial dose was given at 1–2 months old.

Specimen tested, assay used	Response	Shed virus	No response	Shed virus
Serum				
RSV neutralizing antibody	0	0	22	4
IgA ELISA for RSV F protein	9	0	13	4
IgA ELISA for RSV G protein	18	0	4	4
IgG ELISA for RSV F protein	0	0	20	4
IgG ELISA for RSV G protein	1	0	19	4
Nasal wash				
RSV neutralizing antibody	0	0	9	1
IgA ELISA for RSV F protein	3	0	13	1
IgA ELISA for RSV G protein	8	0	7	1

NOTE. Data are no. of vaccine recipients with indicated antibody response to first dose who shed virus after the second dose.

the correlation of increase in antibody titer at 4–8 weeks after immunization with dose of vaccine and level of preexisting maternal antibodies in the infants <24 months old showed that preexisting neutralizing antibodies inhibited or masked an increase in neutralizing antibodies (odds ratio [OR], 4.0; 95% CI, 1.7–9.5; $P = .035$). IgG antibody responses to the F protein ($P < .001$) and the G protein ($P < .001$) were significantly decreased or masked in a direct relationship to the level of preexisting homologous antibodies but were not influenced by age. The frequency (OR, 0.85; 95% CI, 0.72–0.99; $P = .03$) and magnitude ($P = .02$) of IgA responses to F, but not to G, were significantly depressed by maternally acquired IgG antibody level to the homologous protein but not by age.

Evidence for lack of transmission of cpts-*248/404.* Vaccine and placebo recipients often came into close contact with one another for brief periods during follow-up examinations. However, virus was not recovered from placebo recipients, and only 1 of 52 seronegative control subjects developed a serological response. Transmission had been shown under similar circumstances with earlier vaccines in this lineage [25]. A more formal demonstration of the level of transmission will be performed with this and future candidates in a day care setting, as described elsewhere [23].

Effect of breast-feeding on magnitude of virus shedding. Seventeen of 18 children who were exclusively breast-fed shed virus with a mean peak titer of $10^{4.5}$, whereas 7 of 12 exclusively bottlefed shed virus with a lower mean peak titer of $10^{3.5}$.

Stability of ts *and attenuation phenotype of the vaccine.* Of the 176 specimens tested, 173 had a level of temperature sensitivity of plaque formation within 1°C of that of the *cpts*-248/404 vaccine virus, which indicates the high level of phenotypic stability of this virus after replication in susceptible infants and children. Specimens obtained from a single 11-week-old vaccine recipient on days 15, 16, and 17, however, contained an RSV isolate that produced plaques at 38°C, which is 2°C higher than the highest temperature at which *cpts*-248/404 produces plaques. This vaccine recipient experienced a pattern of congestion similar to that seen in other infants receiving this vaccine. Virus from these 3 nasal wash samples was amplified at permissive temperature (32°C), and isolates then were amplified further at both 32°C and 37°C. The higher shut-off temperature of these amplified virus suspensions was confirmed, and the recovered virus was shown to be of vaccine origin by nucleotide sequence analysis. The nucleotide sequences of the genomic regions of these recovered viruses that contain the attenuating mutations present in *cpts*-248/404—namely, the set of 5 *cp* mutations [29], the 248 mutation [30], and the 404 mutation in the M2 gene start sequence [31]—were determined. The recovered virus was found to have sustained a single C→A nucleotide substitution at nucleotide position 9 of the M2 gene start sequence. The mutation in the recovered vaccine occurred at the same position that specifies the 404-M2 attenuating mutation (table 4). It is interesting to note this substitution is not a reversion to the wt RSV T nucleotide at this position.

To assess the effect of this nucleotide substitution on the attenuation phenotype of the resulting virus, virus isolated from the vaccine recipient on day 15 was studied in mice and compared with wt RSV A2, *cpts*-248 (the parent of *cpts*-248/404), and *cpts*-248/404 viruses that were evaluated at the same time in mice. The viruses from the vaccine recipient replicated to the same level in the upper and lower respiratory tract of mice as *cpts*-248, which indicates that the loss of the distinguishing M2-404 mutation returned the virus to the same level of attenuation as *cpts*-248 (table 4). In a previous study, a recombinant virus counterpart of *cpts*-248/404, constructed by site-directed mutagenesis to lack the M2-404 mutation, was shown to have the same temperature sensitivity and attenuation phenotype as *cpts*-248 virus [27].

Surveillance. To examine whether infection with live attenuated *cpts*-248/404 RSV vaccine modified the frequency or severity of wt RSV–associated illness, young infants and seronegative children (i.e., RSV-naive individuals) immunized in these trials and age-matched control subjects in the same clinical care setting were closely followed through the subsequent RSV epidemic season. As in previous studies with live attenuated RSV vaccines, enhanced illness did not occur during reinfection of vaccine recipients with wt RSV [22, 25].

Indeed, the surveillance provided preliminary evidence that the live attenuated RSV vaccine induced a measure of protection (table 5). The rate of subsequent wt RSV infection, as judged by a serological increase, was very high and did not differ between 6–24-month-old vaccine recipients and age-matched control subjects, with 37% overall becoming infected. Indeed, in those <6 months old, there was a significantly higher seroconversion rate in the vaccine recipients than in the age-matched control subjects, which we suggest might result from the vaccine priming the immune system to mount a booster response. There was a marginally significant lower rate of children with subsequent symptomatic wt RSV infection in 6–24-month-old vaccine recipients (4%) than in age-matched control

Table 4. Respiratory syncytial virus (RSV) shed by a vaccine recipient on days 15, 16, and 17.

Variable	Mutation			Virus titer at indicated temperature, $\log_{10}$ pfu/mL					Mean titer in mice,[a] $\log_{10}$ pfu/g tissue		Nucleotide[b]							
											cp					248	404-M2[c]	404-L
	cp	248	404-M2	32°C	36°C	37°C	38°C	39°C	Nasal turbinate	Lung	1938	6313	7228	9453	13565	10989	7605	12046
Compared virus																		
wt RSV A2	−	−	−	5.2	5.0	5.1	5.1	5.2	4.5	4.3	G	A	C	G	C	A	T	T
cpts-248	+	+	−	6.4	6.4	6.1[d]	≤0.7	<0.7	3.4	3.1	A	C	T	A	T	T	T	T
cpts-248/404	+	+	+	4.9	3.6[d]	≤0.7	<0.7	<0.7	2.0	2.4	A	C	T	A	T	T	C	A
Day RSV recovered																		
Day 15	+	+	−	6.5	6.3	6.1	6.0[d]	≤0.7	3.6	3.3	A	C	ND	A	ND	ND	A	ND
Day 16	+	+	−	6.4	6.2	6.1	6.0[d]	≤0.7	ND	ND	A	C	ND	A	ND	ND	A	ND
Day 17	+	+	−	6.5	6.3	6.0	6.0[d]	≤0.7	ND	ND	A	C	ND	A	ND	ND	A	ND
Passage temperature[e]																		
32°C	+	+	−	6.3	6.1	6.1	6.0[d]	≤0.7	3.6	3.5	A	C	T	A	T	T	A	A
37°C	+	+	−	5.5	4.9	5.0	4.8[d]	≤0.7	3.4	3.3	A	C	T	A	T	T	A	A

NOTE. RSV was recovered from a single vaccine recipient on days 15, 16, and 17 and was passaged twice on HEp-2 cells at 32°C. Virus has a nucleotide substitution at the 404 mutation site in the M2 gene start signal, which reduced its temperature sensitivity and attenuation in mice to a level similar to that of RSV *cpts*-248. Shut-off temperature is defined as the lowest restrictive temperature at which a ≥100-fold reduction of plaque titer is observed and is underlined. *cp*, cold-passaged; ND, no data; wt, wild type; +, presence of mutation; −, absence of mutation.

[a] Groups of 5 mice under light anesthesia were given 10^6 pfu of the indicated virus in a 0.1-mL inoculum. After 4 days, virus titer was determined in the nasal turbinate and lung tissues.

[b] Numbered from 3′ end of negative-sense (viral) RNA. Nucleotide assignments are given in the positive sense.

[c] M2 gene start signal (nucleotide 7605 is underlined): A2 wt, GGGGCAAATA; *cpts*-248/404, GGGGCAAACA; and isolate, GGGGCAAAAA.

[d] Pin-point plaque size.

[e] Virus present in the day 15 nasal wash sample was grown at either 32°C or 37°C on HEp-2 cells. Five plaques were picked at each temperature, and passage was continued at either 32°C or 37°C. Results are shown for only plaque no. 4 at each temperature, although remaining plaques were tested and had the same level of temperature sensitivity and the same nucleotide sequence as those shown here.

subjects (20%; table 5). There were too few LRIs to reach any conclusions of vaccine efficacy.

Discussion

The strategy of passaging virus at low temperature to generate vaccine candidates has been successfully used to derive topically administered vaccines for influenza [32] and parainfluenza type 3 [33] that are appropriately attenuated, immunogenic, genetically stable, and, as shown in the case of influenza, highly efficacious on exposure to wt influenza virus [32]. Similar *cp* derivation of a vaccine for RSV yielded a mutant that was only partially attenuated [13]. The current systematic approach to exploring the correlates of virulence and using animal models to determine attenuation and infectivity has been very predictive of behavior of candidate vaccine strains in humans and in establishing a rank-order attenuation of such vaccine mutants for the young seronegative child.

This is the first time that a live attenuated RSV vaccine candidate has been evaluated in 1–2-month-old infants, the target age for vaccination, which allows us to examine factors that influence safety, infectivity, and immunogenicity of an RSV vaccine in early infancy. The demonstration that *cpts*-248/404 retained sufficient residual virulence to cause mild symptomatic URI in 1–2-month-old infants and to readily infect the respiratory tract and to attain a titer $\geq 10^4$ pfu of virus per milliliter of nasal wash would not have been predicted from the chimpanzee studies [19, 20]. Therefore, the young infant is a more permissive host for RSV than the chimpanzee. The absence of lower respiratory tract illness in the vaccine recipients is consistent with the failure to recover the vaccine candidate from the chimpanzee's trachea and the shut-off temperature of *cpts*248/404, which is less than that of human core body temperature, 37°C.

The level of passively acquired maternal antibodies and the infant's age did not have an effect on replication of this attenuated RSV strain in the nasopharynx. This observation is similar to data with bovine parainfluenza [34] and rotavirus vaccines given at the same age [35] and indicates that the mucosal route of administration of a live attenuated vaccine virus permits replication in the upper respiratory tract or gastrointestinal tract, despite the presence of a moderately high titer of serum antibodies. Very high levels of infused RSV serum antibodies can limit upper respiratory tract replication in experimental animals, but this is not an efficient process. Also, it should be noted that the effectiveness of high-titered RSV antibody in prophylaxis against human disease is presumed to be primarily the result of passive transfer of antibody across the mucosal epithelium into the lumen of the lower respiratory tract [36]. Our study also suggests that breast-feeding does not interfere with RSV replication, although breast milk may have antibodies to RSV [37].

In contrast, maternal antibodies and/or age had an inhibitory effect on neutralizing antibody responses and inhibited or masked RSV IgG-binding antibody responses. The vaccine recipients in the youngest age group rarely developed serum antibody re-

Table 5. Surveillance of *cpts*-248/404 vaccine recipients and unvaccinated control subjects during the subsequent respiratory syncytical virus (RSV) epidemic.

Age at immunization, subject	*n*	No. (%) who developed RSV-associated illness[a]				Serological response	
		Any	URI	Otitis media	LRI	Total	RSV positive
<6 Months							
Vaccine recipients[b]	28	6 (21)	5 (18)	2 (7)	0 (0)	25	12[c]
Unimmunized age-matched controls[d]	51	14 (28)	13 (26)	5 (10)	2 (4)	37	4
6–24 Months							
Vaccine recipients[b]	28	1 (4)[e]	0 (0)[f]	1 (4)	1 (4)	24	7
Unimmunized age-matched controls[d]	70	14 (20)	9 (13)	6 (9)	1 (1)	52	21

NOTE. LRI, lower respiratory tract infection; URI, upper respiratory tract infection.

[a] Culture positive at time of illness. Serological response is ≥4-fold increase in neutralizing antibody.

[b] One vaccine recipient 6–24 months and 4 vaccine recipients <6 months were not included in surveillance because they had no evidence of vaccine infection.

[c] $P < .01$ when comparing serological responses in vaccine recipients and control subjects.

[d] Age-matched participants were <9 months old at the beginning of the winter surveillance (i.e., were RSV naive).

[e] $P = .059$ when comparing total illness in vaccine recipients and control subjects.

[f] $P = .056$ when comparing URI in vaccine recipients and control subjects.

sponses that could be detected by a sensitive plaque-reduction neutralization assay or an IgG ELISA assay to purified F and G protein. This lack of a detectable serum response is not unique to vaccine-induced immunity. As we report in this article and as has been documented elsewhere [38], <50% of young infants hospitalized with culture-documented wt RSV infection demonstrate a neutralizing response. It is also possible that a low-level IgG response is masked by residual maternal antibody, since the level of maternal IgG antibodies to RSV F and G proteins in the 1–2-month-old infants is comparable with that seen in the 6–24-month-old children after vaccination, but vaccination did not decrease the expected decay of maternally acquired RSV IgG antibodies, and neutralizing antibody titers were low enough that an increase might have been detected. Passive transfer of RSV antibodies in animals suppress the immune response to RSV F and G proteins expressed by vaccinia virus, despite the fact that the virus vector is not inhibited in its growth. These antibody-suppressed animals are susceptible to RSV reinfection [39]. Previous studies in human infants infected with wt RSV virus have suggested that both age, acting primarily on F protein responses, and the level of maternal antibodies, acting primarily on G protein responses, influence the response to infection with wt RSV [11]. Multivariate analysis suggested that, in this study, the suppression of the all IgG responses and the IgA antibody response to F from the vaccine virus were a function of the level of maternal acquired antibodies and not of age.

Serum IgA responses to the RSV G protein proved to be the most consistent response in the youngest infants. It is reasonable to suggest that the IgA serum antibody is a direct result of replication of vaccine virus in the mucosa of the respiratory tract and reflects a response that originated in the mucosal arm of the immune system. Mucosal IgA responses to RSV F and G protein were also seen, although with a lower frequency than that seen in serum, again more commonly to the G protein. IgA in the respiratory tract is inherently more difficult to measure, because of collection methodology and rapid clearance of IgA in secretions. The IgA responses were not accompanied by a measurable increase in nasal wash or serum neutralizing antibody in the 1–2-month-old vaccine recipients.

The unexpected focus of the infant's immune system on the RSV G protein strongly suggests that a live attenuated RSV vaccine will need to be a bivalent vaccine that contains RSV subgroups A and B, because the G proteins of the RSV subgroups are only ~50% related by amino acid sequence and only 5% related antigenically [40].

The *cpts*-248/404–infected vaccine recipients were highly resistant to infection with the second dose of vaccine. Vaccine virus was not recovered from any vaccine recipient who developed any immunological response to the first dose of vaccine. The second dose of vaccine induced a slight boost in immunity in vaccine recipients infected with the first dose but did not lead to the enhanced neutralizing antibody response seen in the chimpanzee model [19]. Because the second dose of vaccine did infect 2 of 3 children not infected by the first dose, an argument can be made for 2 doses of vaccine as a minimum schedule for immunization.

Each of the vaccine recipients protected against a second dose of vaccine developed a serum IgA response to the G protein after the initial dose, whereas all those infected with the second dose failed to mount such a response to the initial dose. Immunity induced by vaccinia virus that expresses RSV G has been demonstrated, although the degree of protection was less than that seen with a vaccinia virus F recombinant [41]. It is possible that the protection afforded in the present study by IgA antibodies to the RSV G protein were mediated by neutralizing IgA antibodies below the level of our detection, but it is also possible that mucosal IgA antibodies are mediating protection by a mechanism other than, or in addition to, viral

neutralization. Proposed mechanisms of antiviral action for IgA beyond neutralization include antibody-dependent cellular cytotoxicity [42] and intracellular interruption of virus replication during trancytosis of IgA across the epithelial mucosa [43]. The critical response may be mucosal IgA memory on secondary challenge, as reported for RSV in calves whose primary responses are inhibited by colostrums [44]. Others have reported cell-free and cell-bound IgA antibody in the nasopharynx in the course of primary RSV infection in children and its lack of correlation with neutralizing activity [45]. Work with other viral model systems has suggested that protection may be seen in the absence of a neutralizing antibody response that is mediated by components of the cellular immune response measured by lymphoproliferation, as shown in monkeys infected with measles [46]. An advantage of using a live attenuated vaccine is that it can theoretically stimulate a balanced immune response, including antibodies, MHC class I restricted $CD8^+$ cytotoxic T cell response, and a $CD4^+$ T cell memory.

Our inability to infect seropositive children with the attenuated *cpts*-248/404 is another indication of RSV immunity. The marked inhibition of vaccine virus replication by prior natural infection is similar to that seen with influenza and parainfluenza type 3 viruses, and, collectively, they are a strong indicator that effective immunity to respiratory viruses can be induced. It is, however, important to emphasize that the resistance observed to vaccine challenge was to an attenuated virus. Finally, the vaccine recipients 6–24 months old had decreased RSV-associated illness on subsequent exposure to wt RSV. The observed decrease in subsequent wt RSV illnesses by vaccine recipients is an encouraging signal that a similarly derived RSV vaccine may prove to be efficacious when tested for protection against LRI. It is clear from serological studies that wt RSV reinfection occurs frequently in vaccine recipients, as it does after natural infection, and thus cannot serve as a marker for vaccine efficacy.

Despite the high level of replication in fully susceptible young vaccine recipients, *cpts*-248/404 maintained the full *ts* phenotype in 173 of 176 isolates; however, 3 isolates from 1 vaccine recipient exhibited a decrease in their level of temperature sensitivity and in their attenuation in rodents. The 3 virus isolates retained the set of 5 *cp* mutations and the 248 mutation in the L protein of RSV and exhibited a level of temperature sensitivity and attenuation for rodents that were characteristic of viruses that contain these mutations. The alteration in the level of temperature sensitivity and partial loss of attenuation for rodents resulted from a single nucleotide substitution in the M2 gene start sequence at the same position as the original attenuating mutation. As indicated below, a more attenuated RSV vaccine than *cpts*-248/404 is being sought. It is anticipated that the introduction of an additional attenuating mutation will result in a concomitant increase in the stability of the attenuation phenotype.

The *cpts*-248/404 vaccine is not an acceptable RSV vaccine candidate for the youngest infant. It might have a role in seronegative children >6 months old to prevent the substantial burden of RSV-associated URI, otitis media, and milder LRI seen at that age. For the 1–2-month-old infant, ≥1 additional attenuating mutations need to be introduced in *cpts*-248/404 to generate a vaccine that is slightly more attenuated. This can be readily achieved using reverse genetics [47, 48]. Equally important to the optimization of an RSV vaccine will be an understanding of the immune mechanisms by which resistance to reinfection with vaccine virus is conferred in the young child. Future studies will investigate whether protection against a second dose of vaccine continues to translate into protection against reinfection with wt virus and/or the amelioration of the severity of illness because of RSV in the young child, as seen in this study.

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